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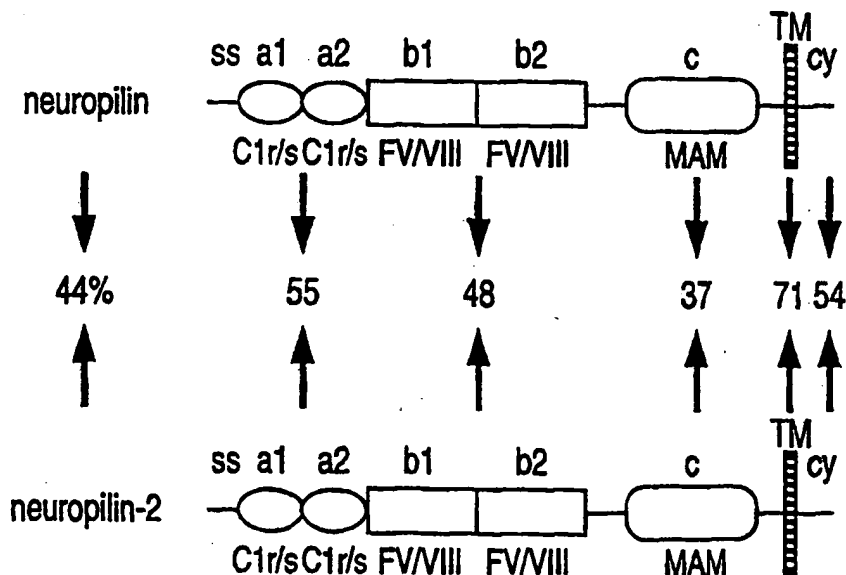
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(54) Title: SEMAPHORIN RECEPTOR

(57) Abstract

The semaphorin family contains a large number of phylogenetically conserved proteins and includes several members that have been shown to function in repulsive axon guidance. Semaphorin III (Sema III) is a secreted protein that *in vitro* causes neuronal growth cone collapse and chemorepulsion of neurites, and is required *in vivo* for correct sensory afferent innervation and other aspects of development. The mechanism of Sema III function, however, is unknown. Here, we report that neuropilin, a type I transmembrane protein, is a Sema III receptor. We also describe the identification of neuropilin-2, a related neuropilin family member, and show that neuropilin and neuropilin-2 are expressed in overlapping, yet distinct, populations of neurons in the rat embryonic nervous system.



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SEMAPHORIN RECEPTOR

The applicants claim the benefit of provisional application Serial No. 60/052,762, filed July 17, 1997, which application is expressly incorporated herein.

TECHNICAL FIELD OF THE INVENTION

This invention is related to the field of developmental biology. In particular it is related to the area of axon guidance cues.

BACKGROUND OF THE INVENTION

The complex wiring of the adult nervous system is dependent upon the occurrence during neurodevelopment of an ordered series of axon guidance decisions that ultimately lead to the establishment of precise connections between neurons and their appropriate targets. These guidance events can act over long or short distances, and they can be either attractive or repulsive in nature (Tessier-Lavigne and Goodman, 1996). An important first step in elucidating the mechanisms by which long-distance chemotropic cues mediate axon guidance is identification of the receptors that bind these cues. Identification of two phylogenetically conserved gene families, the semaphorins and the

netrins, has advanced our understanding of the cellular and molecular basis of long-range influences on axon guidance. Semaphorins and netrins function as chemotropic cues for specific populations of neurons during development (Keynes and Cook, 1995). The netrins have been implicated in long-range attractive and repulsive guidance events in *Caenorhabditis elegans* (UNC-6), vertebrates (netrin-1 and netrin-2), and *Drosophila* (netrin-A and netrin-B) (Serafini et al., 1994; Varela-Echavarría et al., 1997). Genetic studies in both invertebrates and vertebrates, and biochemical studies in vertebrates, show that two immunoglobulin (Ig) superfamily subgroups, one including the Deleted in Colorectal Cancer (DCC), UNC-40, and Frazzled proteins, and the other including the UNC-5, UNC5H1, UNC5H2, and RCM proteins, contain netrin receptors involved in mediating attractive and repulsive netrin functions (Tessier-Lavigne and Goodman, 1996; Ackerman et al., 1997; Fazeli et al., 1997; Leonardo et al., 1997). At present, however, semaphorin receptors have not been identified.

The semaphorins comprise a large family of both transmembrane and secreted glycoproteins, suggesting that some semaphorins act at a distance while others act locally (Kolodkin, 1996; Puschel, 1996). Semaphorins are defined by a well-conserved extracellular semaphorin (sema) domain of approximately 500 amino acids. Secreted semaphorins contain an Ig domain that is C-terminal to the sema domain, while

transmembrane semaphorins can have an Ig domain, type 1 thrombospondin repeat, or no obvious domain motif N-terminal to their transmembrane domain. Semaphorins are present in a variety of neuronal and non-neuronal tissues. Their function in neuronal growth cone guidance, however, has been addressed most extensively.

Two secreted semaphorins, vertebrate collapsin-1/Sema III/Sem D (species homologues) and *Drosophila* semaphorin II (D-sema II) (Matthes et al., 1995), have been shown to function selectively in repulsive growth cone guidance during development. Collapsin-1 (Coll-1) was identified in a search for growth cone collapsing factors from the membranes of adult chick brain tissue (Luo et al., 1993). Acute application of recombinant Coll-1 induces the collapse of a subset of dorsal-root-ganglia (DRG) neuron growth cones at sub-nanomolar concentrations, but has no effect on chicken retinal ganglion cell growth cones. Brain-derived membrane extracts enriched for Coll-1 and immobilized to beads, however, provided sensory neurons in culture with a localized repulsive cue capable of steering growth cones away from beads rather than causing complete growth cone collapse (Fan and Raper, 1995).

Genes encoding human, rat and mouse Sema III/Sem D (referred to below as Sema III) were identified based on their similarity to other semaphorins (Giger et al., 1996; Kolodkin et al.,

1993; Messersmith et al., 1995; Puschel et al., 1995). Sema III can act as a chemorepellent for NGF-dependent embryonic (E14) DRG sensory neurons. It has little effect, however, on neurotrophin-3 (NT-3)-responsive E14 DRG sensory afferents. The E14 ventral spinal cord secretes a chemorepellent activity selective for NGF-, not NT-3-, dependent E14 DRG sensory afferents (Fitzgerald et al., 1993; Messersmith et al., 1995; Puschel et al., 1996). This correlates well with the expression pattern of sema III in the ventral cord during the time of sensory afferent innervation, and the segregation of NT-3- and NGF-dependent sensory afferents, respectively, into ventral and dorsal targets in the spinal cord (Messersmith et al., 1995).

Indeed, antibody perturbation of Coll-1 at analogous stages in chick neurodevelopment supports the idea that Coll-1 is the ventral cord repellent (Shepherd et al., 1997). This is further supported by the observation that mice with a targeted deletion of the sema III gene exhibit defects in the trajectories of certain NGF-responsive sensory afferents (Behar et al., 1996). In addition, functional studies show that Sema III can act as a chemorepellent for spinal motor neurons and a subset of cranial motor neurons (Varela-Echavarria et al., 1997). Coupled with extensive analysis of sema III and Coll-1 expression (Giger et al., 1996; Shepherd et al., 1996; Wright et al., 1995), all of these data suggest that specific populations of embryonic and adult neurons

require Sema III for establishment, and possibly maintenance, of their appropriate patterns of connections. The rapid response of DRG growth cones in culture to Coll-1 and Sema III, and the low concentrations of these factors needed to elicit a response, strongly suggest that a receptor-mediated signal transduction mechanism underlies the action of these proteins on the cytoskeletal reorganization events that ultimately influence growth cone guidance.

SUMMARY OF THE INVENTION

It is an object of the present invention to provide a method of monitoring the interaction of a semaphorin and a neuropilin.

It is another object of the present invention to provide an isolated and purified subgenomic nucleic acid molecule which encodes a new mammalian neuropilin.

It is an object of the present invention to provide a method of identifying axon guidance cues.

Another object of the invention is to provide a method for monitoring the interaction between a semaphorin and a neuropilin so that agonists and antagonists can be identified.

Another object of the invention is to provide a polypeptide useful for antagonizing the interaction between a semaphorin and a neuropilin.

Another object of the invention is to provide a polypeptide useful for antagonizing the interaction between a semaphorin and a neuropilin.

It is still another embodiment of the invention to provide an antibody preparation useful for isolating and detecting a neuropilin protein.

It is yet another object of the invention to provide an isolated and purified mammalian neuropilin protein.

These and other objects of the invention are achieved by one or more of the embodiments described below. In one embodiment a method of monitoring the interaction of a semaphorin and a neuropilin is provided. The method comprises the steps of:

contacting a first protein comprising an extracellular domain of a neuropilin with a second protein which comprises an extracellular domain of a semaphorin under conditions where the extracellular domain of the neuropilin binds to the extracellular domain of the semaphorin;

determining the binding of the first protein to the second protein or second protein to the first protein.

According to yet another embodiment an isolated and purified subgenomic nucleic acid molecule is provided. The molecule encodes a mammalian neuropilin-2 and has at least 90% sequence identity to SEQ ID NO: 1.

In yet another embodiment of the invention a method of identifying axon guidance cues is provided. The method comprises the steps of:

contacting a detectably labeled mammalian neuropilin protein or semaphorin-binding portion thereof with a mixture of proteins secreted by neuronal cells;

removing proteins of the mixture which do not bind to the detectably labeled mammalian neuropilin; wherein a protein of the mixture which binds to the detectably labeled mammalian neuropilin or said portion is identified as a candidate axon guidance cue.

According to another aspect of the invention a method is provided for monitoring the interaction between a semaphorin and a neuropilin. The method comprises the steps of:

contacting a fusion protein comprising a semaphorin sema or Ig basic domain with cells which express a neuropilin;

detecting the fusion protein comprising the semaphorin sema or Ig basic domain which binds to the cells.

As another aspect of the invention a method is provided for monitoring the interaction between a semaphorin and a neuropilin. The method comprises the steps of:

contacting a protein comprising a semaphorin sema or Ig basic domain with cells which express a polypeptide comprising an extracellular domain of a neuropilin;

detecting the protein comprising the semaphorin sema or Ig basic domain which binds to the cells.

As still another aspect of the invention a polypeptide portion of neuropilin useful for antagonizing the interaction between a semaphorin and a neuropilin is provided. The polypeptide comprises the extracellular domain of a neuropilin.

In yet another embodiment of the invention an isolated and purified protein which is neuropilin-2 is provided. The amino acid sequence of neuropilin-2 is shown in SEQ ID NO: 2. Other neuropilins having at least 90% amino acid identity with SEQ ID NO: 2 are also provided.

According to still another aspect of the invention a method of monitoring the interaction between a semaphorin and a neuropilin is provided. The method comprises the steps of:

coculturing in a matrix (a) embryonic nerve cells with (b) cells which have been transfected with an expression construct encoding a semaphorin and which express the semaphorin;

adding to the cells an inhibitor of binding of the semaphorin and the neuropilin;

determining the inhibition of embryonic nerve cell axon outgrowth adjacent to the cells which express the semaphorin in the presence and absence of inhibitor.

As another aspect of the invention a method is provided for monitoring the interaction between a semaphorin and a neuropilin. The method comprises the steps of:

culturing embryonic nerve cells under conditions in which they display growth cones;

contacting the embryonic nerve cells with a semaphorin and an anti-neuropilin antibody;

observing the effect of the antibody on the collapse of the growth cones.

Yet another aspect of the invention is provided by an antibody preparation which specifically binds to a neuropilin protein. The antibody does not specifically bind to neuropilin-1. Particular antibodies bind exclusively to the extracellular domain.

An additional embodiment of the invention is an isolated and purified mammalian neuropilin protein which comprises a signal sequence, two complement binding domains, two coagulation factor domains, a MAM domain, a transmembrane domain and a cytoplasmic domain, with the proviso that the neuropilin protein is not neuropilin-1.

The invention thus provides the art with potent modulators of nerve cell growth, immune responsiveness, and viral

pathogenesis, which can be used in the treatment and diagnosis of neurological disease and neuro-regeneration, immune modulation including hypersensitivity and graft-rejection, and diagnosis and treatment of viral and oncological infection/diseases.

The neuropilins, neuropilin-encoding nucleic acids, and unique portions thereof also are useful in screening chemical libraries for regulators of semaphorin-mediated cell activity, in genetic mapping, as probes for related genes, as diagnostic reagents for genetic, neurological, immunological, and oncological disease and in the production of specific cellular and animal systems for the development of neurological, immunological, oncological and viral disease therapy.

The medical applications of such compounds, their agonists, and their antagonists are enormous and include modulating neuronal growth regenerative capacity, treating neurodegenerative disease, and mapping (*e.g.*, diagnosing) genetic neurological defects.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A to 1F. Sema-AP binds to neuropilin. COS cells were transfected with an expression vector encoding neuropilin (Figs. 1A-1C) or the empty vector (Fig. 1D). After two days, cells were incubated with Sema-AP (Fig. 1A) or SEAP (Fig. 1B) and then processed for alkaline phosphatase activity, or cells were fixed and subjected to immunocytochemistry using anti-neuropilin IgG (Figs. 1C and 1D). No neuropilin immunoreactivity was detected

when COS cells expressing neuropilin were incubated with pre-immune IgG. (Fig. 1E) Anti-neuropilin immunoblot analysis of whole cell extracts prepared from COS cells that were transfected with the empty expression vector (lane 1) or an expression vector encoding neuropilin (lane 2). (Fig. 1F) Sema-AP binds directly to the extracellular domain of neuropilin. Either Sema-AP or SEAP was incubated with soluble myc-tagged neuropilin extracellular domain (myc-neuropilin^{ex}). Then, myc-neuropilin^{ex} was immunoprecipitated with an antibody directed against the myc epitope, and alkaline phosphatase activity in the immune complex was determined as described in the Examples. AP activity was measured as described in the Examples and is reported as OD₄₀₅/second. Shown are the means +/- SEM of three independent experiments. Scale bar = 25 μ m.

Figures 2A-2G. Sema-AP binding sites and neuropilin are co-expressed on growth cones and axons of Sema III-responsive neurons. Dorsal root ganglia (DRG) explants obtained from E14 rat embryos were grown in tissue culture for two days in the presence of NGF, then processed for in situ Sema-AP binding (Figs. 2A and 2C), SEAP binding (Figs. 2B and 2D), or immunocytochemistry with either anti-neuropilin IgG (Fig. 2E) or pre-immune IgG (Fig. 2F). Note that both Sema-AP binding activity and anti-neuropilin immunoreactivity are detected on axons

and growth cones of DRG neurons. (Fig. 2G) Extracts from E14 DRG and spinal cord subjected to immunoblotting using both pre-immune and immune IgG. A single band of ~130 kDa was detected with immune but not pre-immune IgG. The ~40kDa band is likely to be a neuropilin degradation product. Scale bar = 100 μ m, A, B, E, and F; 25 μ m, C, D.

Figures 3A-3B. Scatchard analyses of Sema-AP bound to COS cells expressing neuropilin and to DRG sensory neurons. Sema-AP binding analyses were performed with COS cells that were transfected with a neuropilin expression vector (Fig. 3A), or primary cultures of dissociated rat embryonic DRG neurons (Fig. 3B). Non-specific Sema-AP binding was less than 10% of total binding as measured by Sema-AP binding to untransfected COS cells. Binding characteristics for the experiments shown were as follows: COS cells expressing neuropilin had approximately 125,000 Sema-AP binding sites per cell, and the $KD = 1.5 \times 10^{-9}$. Dissociated DRG neurons had approximately 20,000 Sema-AP binding sites per cell, and the $KD = 0.9 \times 10^{-9}$. Similar results were seen in at least 3 independent COS cell and dissociated DRG binding experiments.

Figures 4A-4H. Neuropilin antibodies inhibit Sema-III-mediated repulsion of NGF- dependent DRG

neurons. (Figs. 4A and 4B) DRG explants were cocultured with COS cells expressing myc-Sema III and grown for 40 hrs. in the absence (Fig. 4A) or presence (Fig. 4B) of anti-neuropilin antibodies (100 μ g/ml IgG fraction). (Fig. 4C) Schematic diagram depicting DRG neurons, COS cells, and parameters measured in experiments presented in (Fig. 4D). P = proximal; D = distal. (Fig. 4D) Quantitation of the effects of anti-neuropilin antibodies on the repulsive activity of Sema III. Shown are the means \pm SEM of axon outgrowth (proximal length/distal length) of DRG neurons in the coculture assay grown in the absence (-Ab) or presence (+Ab) of anti-neuropilin IgG fraction. The degree of axon outgrowth was determined in three separate experiments. Anti-neuropilin antibodies significantly inhibited the repulsive activity of Sema III as determined by a Students T-test ($P < 0.0001$). The average amount of axon outgrowth on lateral sides of the explants as well as the average distance between the explants and the COS cell clumps were not different between the various groups. Although the cocultures for these experiments were grown in the presence of anti-neuropilin or in the absence of rabbit antibodies, additional experiments showed that pre-immune IgG (100 μ g/ml) had no effect on the repulsive activity of Sema III ($P < 0.001$; $n=21$, pre-immune IgG fraction and $n=26$, immune IgG fraction). (Fig. 4E-H) Neuropilin immunoreactivity was specifically detected in neurons previously shown to express

neuropilin mRNA ((Kawakami et al., 1995); Fig. 6). (Fig. 4E) Cross-section of an E14.5 rat spinal cord. Strong neuropilin immunoreactivity was found in DRG (asterisk) and their central and peripheral projections. The dorsal funiculus (DF) and motor axons that leave the ventral horn (arrow; data not shown) display strong neuropilin immunoreactivity. The sympathetic chain ganglion (SG) was stained. (Fig. 4F) No labeling was detected on parallel sections processed with the pre-immune IgG. (Fig. 4G) Parasagittal sections of the head showing strong neuropilin immunoreactivity in the sensory trigeminal ganglion (TG), including the ophthalmic (arrowhead) and maxillary (arrow) branches. A corresponding section stained for neuropilin mRNA by in situ hybridization revealed very strong staining in cell bodies of the trigeminal ganglion. Scale bar = 400 μ m, Figs. 4A and 4B; 300 mm, Figs. 4E and 4F; 180 mm, Figs. 4G and 4H.

Figures 5A and 5B. Comparison of the deduced amino acid sequences of rat neuropilin and Npn-2. (Fig. 5A) Putative signal sequence (dashed line), the two complement binding domains (CUB domains; between the * symbols), the two coagulation factor domains (between the # symbols), the single MAM domains (between the closed circles), and the putative transmembrane domains (solid lines) of neuropilin and neuropilin-2 are indicated. (Fig. 5B) Domain alignment and amino acid identity between rat

neuropilin and rat neuropilin-2; ss = putative signal sequence; a1, a2 = the complement binding domains; b1, b2 = the coagulation factor domains; TM = transmembrane domain; cy = cytoplasmic domain.

Figures 6A-6C. Neuropilin and neuropilin-2 are expressed in distinct populations of cells within the spinal cord and DRG. In situ hybridization of cross-sections of E14.5 rat spinal cord with DIG labeled cRNA probes specific for semaphorin III (Fig. 6A), neuropilin (Fig. 6B), and neuropilin-2 (Fig. 6C). (Fig. 6A) Expression of semaphorin III was restricted to the ventral spinal cord, including the basal plate neuroepithelium. (Fig. 6B) Strong expression of neuropilin was observed in DRG (asterisk), motor pools in the ventral horn, the intermediolateral column (arrowhead) and the dorsal horn. (Fig. 6C) neuropilin-2 expression was detected in motor pools, the ventral horn, intermediate zone, and two dorsally extending stripes at the lateral border of the ventricular zone (small arrow). Roof plate (RP) and floor plate (FP) displayed moderate neuropilin-2 expression. Scale bar= 150 μ m.

DETAILED DESCRIPTION OF THE INVENTION

We have identified a high affinity semaphorin receptor and shown it to be neuropilin, an axonal glycoprotein. Neuropilin has been characterized by Fujisawa and colleagues. Prior to its identification as a semaphorin receptor, the role played by

neuropilin during development was unclear. Our finding, however, demonstrates that neuropilin mediates repulsive guidance decisions. Further, we have found that neuropilin is one of a family of proteins that is expressed differentially in the mammalian nervous system during development.

Recently it has been found that VEGF binds to neuropilin. The binding enhances the signalling achieved by VEGF binding to the KDR receptor. Among other biological effects, VEGF is involved in vascularization of tumors. It is a further discovery of the present invention that overlapping regions of neuropilin bind to semaphorin and VEGF (*i.e.*, the B domains or the coagulation factor domains). Thus semaphorin antagonizes the binding of VEGF to neuropilin. (Similarly, VEGF antagonizes the binding of semaphorin to neuropilin.) Thus semaphorin, or another substance which has similar binding properties, can be used to inhibit vascularization of tumors, and subsequent metastasis. Such substances can be found by the methods disclosed in this application. Semaphorin, neuropilin-binding portions of it, such as the sema domain or the Ig basic domain, or other neuropilin-binding substances, can be administered to tumors by direct administration to the tumor. Alternatively, the substance may be administered systemically. The substance may be targeted, if desired to the tumor using appropriate ligands which bind to the tumor. The substance may be encapsulated in liposomes or other

protective formulations. Polynucleotides encoding semaphorin or a neuropilin-binding portion of it can be delivered to the tumor to inhibit vascularization as well.

A number of methods for monitoring the interaction of a semaphorin and a neuropilin are provided. These can be used to identify agents or conditions which inhibit or enhance the interaction. Such agents may be useful as agonists or antagonists. Two proteins each having at least an extracellular domain sufficient for binding of the other protein, neuropilin and semaphorin, are contacted. The binding of the two proteins occurs in the extracellular domain portions. In particular, the binding occurs in the A and B domains of neuropilin. Either the sema domain or the Ig basic domain of semaphorin can be used to bind to neuropilin. The sema domain requires the A and B domains of neuropilin for binding. The Ig basic domain of semaphorin requires only the B domain of neuropilin to bind. Nonetheless, any portion of the binding partners which is selected from the extracellular portion of the partners and which is sufficient to effect binding with the other binding partner may be used.

After binding has occurred the binding of the two proteins to each other can be determined. For example, one of the two binding partners can be detected immunologically, enzymatically, fluorescently, or radiochemically. This is particularly convenient if one of the two partners had been affixed to a solid support prior to

the step of binding. Suitable solid supports are known in the art and include microtiter dishes, column packing materials, beads, and the like. An ELISA format is particularly convenient. Binding can be determined by quantitative or qualitative methods. Either or both of the bound partners can be determined, or the amount which does not bind can be determined.

Alternatively, after binding has occurred an antibody can be used to immunoprecipitate the protein binding complex formed. The antibody may bind to either of the two binding partners as the antigen. The presence of the non-antigen binding partner is determined in the immunoprecipitate. Only if the two proteins bind will the non-antigen be found in the immunoprecipitate. The presence of the non-antigen binding partner in the immunoprecipitate can be detected by any means known in the art. These include analysis on SDS-polyacrylamide gels and immunoassay with an antibody to the opposite binding partner to that used for the immunoprecipitation. The proteins used in the method may be fusion proteins, isolated portions of the binding partners, or the full-length binding partners. As each of semaphorin and neuropilin define gene families, any semaphorin and any neuropilin may be used as binding partners. The proteins may desirably lack a transmembrane and an intracellular domain. One of the two proteins may be "tagged" with an unrelated protein sequence, for easy manipulation and recognition. For example,

myc sequences can be covalently appended to the binding partner, preferably by genetic engineering, to form a binding partner which can be detected and/or purified using an anti-myc antibody. Fusion proteins may conveniently comprise a second protein which has a desirable feature or biological activity, such as an enzymatic activity, or a binding activity. One such fusion protein which has been particularly useful contains alkaline phosphatase, which produces a colored product upon conversion of a suitable substrate, such as NBT and BCIP. Other such enzymes as are known in the art can be readily be used.

Test compounds can be added to one or both of the binding partners in the binding reaction, either before, during, or after the binding reaction. The effect of a test compound on the binding reaction can be used to identify agents useful as agonists or antagonists.

Another variation on the monitoring of the binding interaction involves the use of cells which express a neuropilin. Cells can be tested for expression of a neuropilin using antibodies or any other means known in the art. Cells which have been transfected with a gene construct such that they express a neuropilin can also be used. Using such transfectants, one is not limited to use of cells which naturally express neuropilins. Moreover, using such constructs, one has an excellent null-binding control in the non-transfected cells. Since the neuropilins are expressed on the

surface of cells, there is no need to lyse or otherwise treat or permeabilize the cells prior to binding. In this variation, use of a fusion protein comprising an enzyme and a semaphorin is particularly convenient. See examples, below.

Another variation on the method used to monitor the binding interaction of semaphorins and neuropilins is a two-cell assay. In such an assay two cell types are co-cultured in a matrix which limits diffusion of secreted products. Suitable matrices for growth of cells are known in the art and include collagen matrices. DRG cells can be used as the neuropilin expressing cells. The semaphorin-secreting cells can be cells transfected with a semaphorin expression construct. An inhibitor of the binding interaction is added to the culture and the inhibition of axon outgrowth adjacent to the semaphorin-secreting cell is determined. Outgrowth of axons can be observed under a microscope directly or can be immunostained prior to observation to enhance visibility of axon processes. One inhibitor which can be used is an anti-neuropilin antibody. Anti-(MAM and B) domain antibodies have been found to function as such an inhibitor. Other inhibitors which can be used include polypeptides which comprise an extracellular domain of a neuropilin, preferably comprising a B domain and/or an A domain. Testing the ability of test compounds to affect the binding interaction is also contemplated. Such testing may identify useful agonists and antagonists of axon guidance cuing.

Another method of monitoring the interaction of a neuropilin and a semaphorin is by observing the effect of a semaphorin on growth cones of cultured embryonic neuronal cells, such as DRG cells. Inhibitors of the interaction of neuropilins and semaphorins can be added and their effects on the collapse of growth cones observed. Suitable inhibitors include anti-neuropilin antibodies, anti-B domain antibodies, polypeptides consisting of an extracellular domain of neuropilin. Others may be used as become known. Test compounds can also be added to determine their agonistic or antagonistic capabilities.

According to yet another method of the invention test compounds can be tested to determine if they enhance or decrease the binding of semaphorin to neuropilin. Due to the overlapping binding regions of VEGF and semaphorin on neuropilin, compounds which influence semaphorin binding to neuropilin will also influence VEGF binding. This assay can use whole binding partners or polypeptides comprising binding-sufficient portions.

Axon guidance cues can be identified using the systems disclosed here. A mammalian neuropilin protein, or semaphorin binding portion thereof, can be used as a probe to detect other axon guidance cues. These may include other members of the semaphorin family or other axon guidance cues having other effects. Preferably the neuropilin protein is detectably labeled. A mixture of proteins is contacted with the neuropilin protein and non-bound

proteins are removed, for example by washing, rinsing, and the like. A protein which is found to bind to the neuropilin is identified as a candidate axon guidance cue.

It has been found that neuropilin (as described by Kitsukawa et al.) is a member of a gene family having multiple members. Neuropilin is therefore now called neuropilin-1. A second member of the family is neuropilin-2. This protein is 44% identical overall to neuropilin-1. (See Figure 5.) However, it shows remarkable conservation of its domain structure. Each neuropilin has two complement binding domains (see Bork & Beckman, 1993, *J.Mol. Biol.* 231:539-545), two coagulation factor domains (Toole et al. 1984, *Nature* 312:342-347; Jenny et al. 1987, *Proc. Natl., Acad. Sci. USA* 84:4846-4850; Sanchez, M. et al. (1994) *Proc. Natl. Acad. Sci.* 91:1819-1823; Larocca et al.(1991) *Cancer Res.* 51:4994-4998), a MAM domain (Beckman & Bork, 1993 *Trends Biochem. Sci.* 18:40-41), a single transmembrane domain, and a strikingly short cytoplasmic domain. A subgenomic nucleic acid molecule which encodes neuropilin-2 is provided. The nucleic acid molecule is smaller than the whole chromosome on which the gene naturally resides *in vivo*. The nucleic acid may be purified RNA or purified genomic DNA or cDNA. Other neuropilin-2 molecules which share at least 75%, 80%, 85%, 90%, or even 95% identity with the amino acid sequence or the nucleotide sequence disclosed for rat neuropilin-2 are also contemplated. These can be obtained,

as is known in the art, using such techniques as RT-PCR and degenerate oligonucleotide probe hybridization. The rat and mouse neuropilin-1 molecules are about 98% identical and human neuropilin-1 is about 92% identical to the rat and mouse proteins.

Other neuropilin protein molecules can be obtained functionally, as discussed in the examples, by isolating expression clones which bind to semaphorins.

Also useful are portions of the neuropilin proteins. These can be used to antagonize the interaction between a semaphorin and a neuropilin. The whole extracellular domain of a neuropilin protein can be used or one or more domains of the whole extracellular domain can be used. Suitable domains include the complement binding domains, the coagulation factor domains, and the MAM domain. These may be useful as immunogens for generating inhibitory antibodies. The A and B domain of neuropilin, *i.e.*, the coagulation factor domain, may be useful as a semaphorin (ligand) binding site. Use of the ligand binding site may inhibit semaphorin's biological effects by titrating semaphorin away from the neuropilins which are on the neurons. B (coagulation factor) domains of other non-neuropilins may be useful for this purpose as well.

Antibodies which specifically bind to a neuropilin protein are also useful for inhibition of the neuropilin-semaphorin binding reaction. Polypeptide portions of the neuropilins can be used as

immunogens to raise such antibodies. They may be polyclonal or monoclonal. Techniques for raising such antibodies are well known in the art. Antibodies such as those disclosed below which were raised against the MAM and B domain and which inhibit the biological effect of semaphorins on axonal growth and/or orientation are desirable.

In addition to neuropilin-derived polypeptides and peptides, other prospective agents are screened from large libraries of synthetic or natural compounds. For example, numerous means are available for random and directed synthesis of saccharide, peptide, and nucleic acid based compounds. Alternatively, libraries of natural compounds in the form of bacterial, fungal, plant and animal extracts are available or readily producible. Additionally, natural and synthetically produced libraries and compounds are readily modified through conventional chemical, physical, and biochemical means. See, *e.g.*, Houghten et al. and Lam et al (1991) *Nature* 354, 84 and 81, respectively and Blake and Litzi-Davis (1992), *Bioconjugate Chem* 3, 510.

The subject peptides/polypeptides are "isolated", meaning unaccompanied by at least some of the material with which they are associated in their natural state. Generally, an isolated polypeptide constitutes at least about 1%, preferably at least about 10%, and more preferably at least about 50% by weight of the total protein in a given sample. "Pure polypeptide" is intended to mean at least

about 90%, preferably at least 95%, and more preferably at least about 99% by weight of total protein. Included in the subject polypeptide weight are any atoms, molecules, groups, or polymers covalently coupled to the subject neuropilin/receptor polypeptide, especially peptides, proteins, detectable labels, glycosylations, phosphorylations, etc.

The subject polypeptides may be isolated or purified in a variety of ways known to those skilled in the art depending on what other components are present in the sample and to what, if anything, the polypeptide is covalently linked. Purification methods include electrophoretic, molecular, immunological and chromatographic techniques, especially affinity chromatography and RP-HPLC in the case peptides. For general guidance in suitable purification techniques, see Scopes, R., *Protein Purification*, Springer-Verlag, N.Y. (1982).

The subject polypeptides generally comprise naturally occurring L-amino acids but D-amino acids or amino acid mimetics coupled by peptide bonds or peptide bond mimetics may also be used. Amino acid mimetics are other than naturally occurring amino acids that conformationally mimic the amino acid for the purpose of the requisite neuropilin/receptor binding specificity. Suitable mimetics are known to those of ordinary skill in the art and include beta - gamma - delta amino and imino acids, cyclohexylalanine, adamantylacetic acid, etc., modifications of the

amide nitrogen, the alpha -carbon, amide carbonyl, backbone modifications, etc. See, generally, Morgan and Gainor (1989) *Ann. Repts. Med. Chem* 24, 243-252; Spatola (1983) *Chemistry and Biochemistry of Amino Acids, Peptides and Proteins, Vol VII* (Weinstein) and Cho et. al (1993) *Science* 261, 1303-1305 for the synthesis and screening of oligocarbamates.

The subject neuropilin polypeptides preferably have a "semaphorin-binding specificity" meaning that the subject polypeptide retains a molecular conformation specific to one or more of the disclosed neuropilins and specifically recognizable by a semaphorin, anti-neuropilin antibody, etc.

"Specific binding" is empirically determined by contacting, for example, a neuropilin-derived peptide with a mixture of components and identifying those components that preferentially bind the neuropilin. Specific binding is most conveniently shown by competition with labeled ligand using recombinant neuropilin peptide either *in vitro* or in cellular expression systems as disclosed herein. Generally, specific binding of the subject neuropilin has binding affinity of 10^{-6} M, preferably 10^{-8} M, more preferably 10^{-10} M, under *in vitro* conditions as exemplified below.

The polypeptides may be modified or joined to other compounds using physical, chemical, and molecular techniques disclosed or cited herein or otherwise known to those skilled in the relevant art to affect their semaphorin-binding specificity or other

properties such as solubility, membrane transportability, stability, binding specificity and affinity, chemical reactivity, toxicity, bioavailability, localization, detectability, in vivo half-life, etc. as assayed by methods disclosed herein or otherwise known to those of ordinary skill in the art.

Neuropilin-1 is expressed and functions in Sema-III-responsive tissues. Neuropilin (previously known as A5) was first identified as a membrane-associated glycoprotein expressed in the tectum of *Xenopus laevis* (Takagi et al., 1987). More recent analyses have demonstrated that mammalian, *Xenopus*, and avian neuropilin are present in a number of discrete neuronal populations (Kawakami et al., 1995; Satoda et al., 1995; Takagi et al., 1995). Importantly, neuropilin distribution patterns in the developing mouse nervous system support our conclusion that it is a Sema III receptor. During mouse development, neuropilin is present in several populations of neurons known to be responsive to Sema III, including DRG sensory neurons, post-ganglionic sympathetic neurons, trigeminal motor neurons, and spinal motor neurons (Kawakami et al., 1995; Messersmith et al., 1995; Puschel et al., 1995; Puschel et al., 1996; Shepherd et al., 1996; Takagi et al., 1991; Takagi et al., 1995; Takagi et al., 1987; Varela-Echavarria et al., 1997). Neuropilin is also expressed in many other populations of developing neurons whose

ability to respond to Sema III has yet to be determined. These include several cranial nerve sensory ganglia, primary olfactory neurons, and neurons within the hippocampus and neocortex.

In addition to biochemical evidence and expression patterns, genetic evidence also suggests that neuropilin is a receptor for Sema III *in vivo*. Transgenic mice that overexpress neuropilin and mutant mice with a targeted deletion of the sema III gene have remarkably similar phenotypes, indicating that both neuropilin and sema III are likely to contribute to the morphogenesis of a similar, if not identical, set of tissues (Behar et al., 1996; Kitsukawa et al., 1995). In the nervous system, loss of sema III function and overexpression of neuropilin produce defects in DRG sensory afferent projections in the spinal cord. In addition, neuropilin overexpression results in defasciculation and ectopic sprouting of spinal motor nerves in regions where non-neuronal Sema III is likely to function as a guidance cue (Giger et al., 1996; Wright et al., 1995). Further, sema III mutant and neuropilin overexpressing mice have similar cardiovascular and bone defects. For example, both mutants have grossly abnormal hearts with dilated, thin-walled, right atria. Moreover, the sema III mutant mice and neuropilin overexpressing mice both exhibit improper skeletal development. These phenotypes, coupled with the expression of neuropilin and sema III in these tissues (Giger et al., 1996; Kawakami et al., 1995; Kitsukawa et al., 1995; Wright et

al., 1995), suggest that both gene products function in a common signaling pathway. In addition to providing indirect, yet compelling, support for our conclusion that neuropilin is a receptor for Sema III in vivo, these data show that Sema III and neuropilin influence development of both neuronal and non-neuronal cells.

Neuropilins and semaphorin signaling

Secreted and transmembrane semaphorins are likely to affect neurodevelopment, at least in part, through their influence on repulsive growth cone steering decisions (secreted semaphorins: as described above; transmembrane semaphorins: H.-H. Yu., H. Araj, S. Ralls, and A. K., in preparation). In vitro, application of Coll-1 to NGF-dependent DRG neurons induces collapse of their growth cones, and this event is mediated by changes associated with the actin cytoskeleton within the growth cone. Growth cones exposed to Coll-1-enriched membrane extracts undergo a loss of F-actin at their leading edges relative to their centers that is not accompanied by alterations in intracellular Ca^{2+} levels (Fan et al., 1993; Ivains et al., 1991). Coll-1 induced growth cone collapse is pertussis toxin (PTX) sensitive, though at present it is unclear whether this effect is directly mediated by ADP ribosylation of G proteins (Goshima et al., 1995; Kindt and Lander, 1995). Our finding that neuropilin is a Sema III receptor suggests that Sema III intracellular signaling does not proceed directly through a G protein-coupled mechanism. A gene encoding collapsin response

mediator protein (CRMP-62) was cloned using this strategy and found to be necessary both for mediation of Coll-1-induced inward ion currents in oocytes and for the activity of Coll-1 on DRG neurons (Goshima et al., 1995). CRMP-62 is a member of a family of related intracellular proteins that includes four members variously expressed in the developing and adult rat nervous system, and also the *C. elegans* protein UNC-33 protein, which is required for axonal elongation and fasciculation (McIntire et al., 1992; Wang and Strittmatter, 1996). The mechanism by which CRMP-62 mediates Coll-1 or Sema III effects on DRG growth cones is unknown.

Neuropilin binds Sema III in the extracellular environment of Sema III-responsive growth cones, and it participates, possibly directly, in propagation of the Sema III signal to the intracellular components that influence actin-based changes in growth cone morphology. The extracellular portions of neuropilin and neuropilin-2 consist of three motifs found in other transmembrane proteins: the complement binding domains (CUB), the coagulation factor domains, and the MAM domains. One or all of these domains may be important for semaphorin binding or for other neuropilin functions (Hirata et al., 1993; Takagi et al., 1995). Our finding that anti-neuropilin antibodies generated against the neuropilin (MAM and B) domain inhibited the repulsive activity of Sema III on NGF-dependent DRG neurons suggests that one of

these domains participates in Sema III binding. In fact, structure-function analysis has demonstrated that the A and B domains both contribute to binding.

The mechanism by which neuropilin transmits the Sema III signal to the interior of the growth cone remains unknown. The intracellular domain of neuropilin is short and contains no motifs with obvious catalytic function nor any domains that offer clues regarding the mechanism of Sema III signal transduction. However, because the intracellular domains of neuropilin and neuropilin-2 are similar with respect to both primary sequence and length, it is likely they share a common signaling mechanism. At least two possible mechanisms of neuropilin signaling exist. Neuropilin may function alone to transduce the Sema III signal. In this model, the intracellular domain of neuropilin could have a novel mechanism of coupling extracellular cues to the inside of the cell. For example, CRMP-62 may associate directly with the intracellular domain of neuropilin, and the activity of CRMP-62 may be modulated upon Sema III binding to neuropilin. Alternatively, a distinct unidentified signaling component of a Sema III receptor complex may exist, as is the case for several other ligand/receptor systems including the CNTF receptor (Ip and Yancopoulos, 1996). In this model, neuropilin may be a ligand binding component and a distinct protein(s) may associate with neuropilin and function to transmit a biochemical signal into the

cytoplasm. Future studies will provide insight into the biochemical interactions between neuropilin, CRMP-62, and other signaling molecules that influence cytoskeletal dynamics of growth cones upon encountering Sema III.

Neuropilin and neuropilin-2 define a gene family

Sema III is one member of a large family of phylogenetically conserved proteins with diverse patterns of neuronal and non-neuronal expression (Kolodkin, 1996; Puschel, 1996). It is likely that these proteins participate in many aspects of development. Therefore, it is important to identify receptors for all of the semaphorins in order to begin to determine their mechanisms of action in target cells. Our identification of neuropilin-2 provides evidence for the existence of a family of neuropilin receptors, and for a model in which at least two neuropilin receptors mediate the cellular responses of semaphorin family ligands.

Neuropilin and neuropilin-2 are closely related proteins that share a common domain structure and a significant degree of amino acid identity throughout their entire length. The structural conservation of these proteins suggests that they share a common function. Neuropilin and neuropilin-2 display unique patterns of expression in the spinal cord, the remainder of the CNS, as well as other non- neuronal tissues. Therefore, it is likely that neuropilin

and neuropilin-2 influence semaphorin-mediated guidance decisions in distinct populations of developing neurons.

The above disclosure generally describes the present invention. A more complete understanding can be obtained by reference to the following specific examples which are provided herein for purposes of illustration only, and are not intended to limit the scope of the invention.

EXAMPLE 1

Neuropilin is a Sema III binding protein

To identify cell surface receptors for Sema III, we used a COS cell expression cloning strategy that employed a Sema III-secreted placental alkaline phosphatase fusion protein (Sema-AP) (Flanagan and Leder, 1990). A COS cell cDNA expression library was constructed using mRNA obtained from rat E14 spinal cord and DRG. cDNAs generated from these mRNAs should encode functional Sema III receptors since Sema III as well as Sema-AP induce collapse of growth cones from NGF-responsive DRG neurons. The cDNA expression library was divided into 140 pools, each containing approximately 750 clones, and cDNA from each pool was transfected into separate wells of COS cells. Two days after transfection, COS cells were fixed, incubated with a solution containing Sema-AP, washed, and then stained for alkaline phosphatase (AP) activity. One positive pool was identified by the presence of a single COS cell with Sema-AP binding

activity. This positive pool of cDNAs was subdivided and re-screened several times until a single cDNA was obtained that conferred Sema- AP binding when expressed in COS cells (Fig. 1A).

Sequence analysis revealed that the Sema-AP binding protein was the full length rat homologue of mouse neuropilin, a protein previously identified and well characterized in mice and other vertebrates (Kawakami et al., 1995). Neuropilin is a type I transmembrane protein that is expressed in a number of populations of neurons, including DRG neurons and spinal motor neurons ((Kawakami et al., 1995); Fig. 2E, 4E). The neuropilin protein consists of a large extracellular domain, a single transmembrane domain, and a short 39 amino acid intracellular domain (Fig. 5). Sema-AP fusion protein bound to neuropilin via its Sema III domain, not the AP domain, because secreted placental alkaline phosphatase (SEAP) alone did not bind to COS cells expressing neuropilin (Fig. 1B). Moreover, Sema-AP binding to COS cells expressing neuropilin was inhibited by myc epitope-tagged Sema III (Sema-myc), and Sema-myc bound directly to COS cells expressing neuropilin but not to untransfected COS cells. Lastly, anti-neuropilin antibodies, directed against a bacterial fusion protein that included the C-terminal MAM domain as well as a portion of the B domain of neuropilin, detected neuropilin in COS cells transfected with a neuropilin expression vector, as shown by

immunocytochemistry (ICC) and immunoblotting (Fig. 1C, E). Together, these results demonstrate that Sema III binds to neuropilin that is expressed on the surface of COS cells.

While our results suggest that neuropilin is a Sema III binding protein, it remained possible that Sema-AP bound to a complex of neuropilin and an endogenous COS cell protein(s), or that neuropilin induced the expression of an endogenous Sema III binding protein in COS cells. Therefore, we next asked whether Sema-AP binds directly to neuropilin in a co-immunoprecipitation assay. For these experiments, an N-terminal myc-tagged neuropilin protein lacking the neuropilin transmembrane and intracellular domains (myc-neuropilin^{ex}) was used to assess whether the extracellular domain of neuropilin can directly interact with Sema III. Myc- neuropilin^{ex} was expressed in COS cells and the tissue culture medium was then incubated with either Sema-AP or SEAP alone. Upon precipitation of the myc-neuropilin^{ex} with a monoclonal antibody directed against the myc epitope, co-precipitation of Sema-AP was determined by the presence of AP activity in the immune complex (Fig. 1F). In contrast, no AP activity above background levels was detected in anti-myc immune complexes collected from samples in which myc-neuropilin^{ex} was incubated with SEAP alone. These results demonstrate that Sema III associates directly with neuropilin.

We next compared the spatial distribution of neuropilin and Sema III binding sites present on the surface of cultured NGF-dependent DRG neurons. Sema-AP binding sites were detected all over the neurons, including their growth cones (Fig. 2A, C). Again, SEAP alone did not bind to DRG neurons, demonstrating that Sema-AP binding was dependent on the Sema III domain, not the AP domain, of the fusion protein (Fig. 2B, D). To assess neuropilin distribution we first subjected extracts of E14 DRG and spinal cord to immunoblotting using our anti-neuropilin antibodies described above. These antibodies detected a single ~130 kDa band which was not observed with pre-immune IgG (Fig. 2G). Using these antibodies, neuropilin immunoreactivity was seen on growth cones, axons and cell bodies of cultured DRG neurons (Fig. 2E).

Generation of Sema-AP, myc-neuropilin^α

To generate the H-Sema III-alkaline phosphatase fusion protein (Sema-AP) expression vector, the human Sema III coding sequences (Kolodkin et al., 1993) were inserted into the Hind III and Bgl II sites of pAPtag-1 (Flanagan and Leder, 1990) to generate a Sema-AP fusion. Then, the entire Sema-AP sequence was excised from the pAPtag-1 vector and inserted into the Hind III and Xho I sites of pCEP4, an expression vector designed to provide high level expression in the EBNA subclone of 293 cells (Invitrogen). Myc epitope-tagged, secreted neuropilin

(myc-neuropilin^{ex}) expression construct was generated as follows: A 2.5 kb fragment of neuropilin lacking the coding determinants of the transmembrane and intracellular domains was obtained by PCR using the entire neuropilin ORF sequence in pcDNA3 (Invitrogen) as a template. The PCR fragment was digested with EcoRI and Xba I and subcloned into a pBluescript vector containing a Kozak consensus sequence, myc epitope tag, and signal sequence originating from peptidylglycine alpha-amidating monooxygenase (PAM) (a gift of Richard Mains and Ruth Marx). This plasmid was digested with Not I, Sal I, and Sca I, and a fragment encoding the Kozak consensus sequence, PAM signal sequence, myc epitope tag, and the entire extracellular domain of neuropilin was isolated. This fragment was then cloned into the Not I and Sal I sites of the pCIneo mammalian expression vector (Promega).

Expression library construction and screening

Polyadenylated RNA isolated from embryonic day 14 rat spinal cord and associated dorsal root ganglia was used to generate cDNA (ZAP-cDNA synthesis Kit; Stratagene). Subsequently, the cDNA was size fractionated and cDNA within fractions containing the largest fragments was ligated into the pMT21 COS cell expression vector (a modified version of pMT2 (Sambrook et al., 1989; Serafini et al., 1994). The ligation products were transformed into E. Coli (ElectroMAX DH10B; Gibco/BRL), and approximately 750 bacterial colonies were grown on 140 separate

plates and harvested to generate 140 pools of cDNAs. Plasmid DNA was isolated from each pool using the Wizard DNA purification system (Promega) and, then, each cDNA pool was independently transfected into COS cells (1X10⁵ cells per 35 mm well of cells) using a Lipofectamine-mediated DNA transfection procedure (Gibco/BRL). Two days after transfection, cells were incubated with Sema-AP, and bound Sema-AP was visualized following an alkaline phosphatase assay done essentially as described (Flanagan and Leder, 1990). Several pools containing one or more plasmids capable of conferring Sema III-AP binding activity were identified, and these pools were used to generate successively smaller plasmid pools followed by transfection and Sema III-AP binding assays. Ultimately, transfection of a single cDNA clone conferred Sema-AP binding activity in transfected COS cells. This clone, encoding rat neuropilin, was sequenced on both strands using the fluorescent di-deoxy terminator method of cycle sequencing on a Perkin Elmer Applied Biosystems Division 373a automated DNA sequencer.

Co-precipitation of Sema-AP and the secreted, extracellular domain of neuropilin

COS cell supernatant containing myc-neuropilin[™], 293 EBNA cell supernatant containing SEAP, or 293 EBNA cell supernatant containing Sema-AP was filter-sterilized and concentrated. Samples containing equal amounts of control

supernatants or supernatants containing myc-neuropilin^{ex} were mixed with samples containing either Sema-AP or SEAP (equal amounts of AP activity). These mixtures were incubated at room temperature for 2 hours. Then, an equal volume of an immunoprecipitation buffer (20 mM Tris, pH 8.0, 140 mM NaCl, 0.5 mM EDTA and 2% Np-40) was added to each mixture, and the samples were centrifuged at 15,000 X g for 15 minutes at 4°C. Supernatants were recovered, and 4 ml of anti-myc antibody (antibody 9E10 ascites fluid) was added to each and samples were incubated with mixing at 4°C for 2 hours. Then, 50ml of protein G sepharose was added to each tube, and immune complexes were collected after one hour. Immune complexes were washed three times with immunoprecipitation buffer, once with PBS, and then the immune complexes were resuspended in PBS. Liquid alkaline phosphatase assays were performed as described above. Background was defined as the amount of AP activity detected in samples in which myc-tagged myc-neuropilin^{ex} was omitted from the sample incubations, and this value was subtracted from all other measurements.

Neuropilin Antisera Production and Immunoblot Analysis

Anti-neuropilin antibodies were produced by immunizing rabbits with a 6-histidine-tagged neuropilin protein which was produced in E. Coli. The bacterial expression construct was made by PCR-amplification of a fragment encoding amino acids 583-856

of rat neuropilin and inserted into the EcoRI and Hind III sites of pTrcHisA (Invitrogen). Expressed protein was purified by immobilized nickel-chelate affinity chromatography. Rabbits were immunized with 375 mg protein in complete Freund's adjuvant, and boosted every two to three weeks with 250 mg protein in incomplete Freund's adjuvant. Serum was collected and the IgG fraction was purified by Protein A Sepharose chromatography. Immunoblot analysis was performed as described (Ginty et al., 1994), using extracts of neuropilin transfected and untransfected COS cells, and E14 DRG and spinal cord.

Immunohistochemistry

E14.5 rat embryos were fixed for 4 hours in ice cold PBS containing 4% paraformaldehyde, and cryoprotected overnight in the same solution containing 15% sucrose. Immunocytochemistry of cryosections (20 μ m) using either immune or preimmune rabbit anti-neuropilin, IgG fraction (0.7 μ g/ml) was done as described (Giger et al., 1996).

EXAMPLE 2

Sema III binds to neuropilin with high affinity

Because Coll-1 elicits biological effects at sub-nanomolar concentrations (Luo et al., 1993), we predicted that a bona fide Sema III receptor should bind to Sema III with high affinity. To determine the affinity of Sema-AP for neuropilin, neuropilin was transiently expressed in COS cells, and whole cell binding analyses

were performed two days later (Fig. 3A). For comparison, the affinity of Sema-AP for its receptor(s) present on NGF-dependent sensory neurons prepared from dissociated E14 DRG was also determined (Fig. 3B). Sema-AP bound to COS cells expressing neuropilin with a high affinity; the calculated equilibrium dissociation constant (KD) was approximately 1.5 nM. There were approximately 125,000 Sema-AP binding sites per COS cell. Interestingly, Sema-AP bound to DRG neurons with an equivalent affinity, and DRG sensory neurons had approximately 20,000 binding sites per cell. These binding affinities are similar to those recently described for netrins and their receptors (Keino-Masu et al., 1996; Leonardo et al., 1997) and Eph receptors and their ligands (Cheng and Flanagan, 1994; Monschau et al., 1997), and they are consistent with a role for neuropilin in Sema III-mediated growth cone collapse and in repulsive guidance of DRG neurons during neurodevelopment. Taken together, these data suggest that neuropilin is a high-affinity Sema III receptor expressed on Sema III- responsive DRG neurons.

Cell surface binding analysis

COS cells were transfected with 2 μ g of pMT21-neuropilin, an expression vector encoding neuropilin, or either no DNA or the empty pMT21 expression vector using Lipofectamine (BRL), recovered in growth media, and then grown for 48 hours prior to binding analysis. Dissociated DRG neurons were cultured from

E14 DRGs. Briefly, DRG neurons were dissociated in a solution containing trypsin (0.05%), the dissociated neurons were washed to remove trypsin and then plated on collagen-coated tissue culture plates (400,000 cells/35 mm plate). Cells were grown in DRG growth medium (88% MEM, 10% FBS, 0.2% glucose, glutamine (2 mM) and NGF (30 ng/ml)) and subjected to binding analysis four days after plating. Quantitative cell surface binding was done essentially as described (Flanagan and Leder, 1990).

EXAMPLE 3

Neuropilin antibodies inhibit Sema III-mediated repulsion of DRG neuron growth cones.

If neuropilin is a receptor for Sema III, then it should be possible to block neuropilin function in NGF- dependent DRG neurons and to prevent Sema III from acting as a repulsive cue. To block neuropilin function, we used our anti-neuropilin antibodies described above. In addition to immunoblotting analysis of extracts of E14 DRG and spinal cord (Fig. 2G), these antibodies were further assessed for specificity by immunostaining sections from E14.5 rat embryos. They specifically reacted with a protein expressed in a subset of neurons including DRG neurons, sympathetic neurons (Fig. 4E), and trigeminal sensory neurons (Fig. 4G). These populations of neurons also express robust levels of neuropilin mRNA as determined by in situ hybridization analysis (Fig. 4H, 6B; (Kawakami et al., 1995)). No immunoreactivity was

detected on tissue sections incubated with IgG purified from pre-immune serum (Fig. 4F). In combination with immunoblot analysis, as well as the expression pattern of the other identified member of the neuropilin family (neuropilin-2, see below), these data strongly suggest that these antibodies specifically recognize neuropilin in DRG neurons.

Coculturing E14 DRG and Sema III-expressing COS cells in a collagen matrix provides a robust assay for the chemorepulsive activity of Sema III on these neurons (Messersmith et al., 1995). As seen previously, NGF-dependent DRG neurons were repelled from COS cells that expressed Sema III (Fig. 4A). Including anti-neuropilin antibodies, however, resulted in an inhibition of the repulsive activity of Sema III (Fig 4B). The amount of axon outgrowth on the side of the DRG adjacent to the Sema III-expressing COS cells was more than two-fold greater in the presence of anti-neuropilin antibodies as compared to cocultures grown in the absence of added antibodies or equal amounts of pre-immune IgG fraction (Fig. 4C and D; $p < 0.0001$). The results of these antibody perturbation experiments indicate that neuropilin activity is required to mediate the repulsive effects of Sema III on NGF-dependent E14 DRG neurons. Since we have shown that neuropilin is a Sema III binding protein, and since neuropilin is expressed on the axons and growth cones of these

neurons, these results demonstrate that neuropilin is an endogenous receptor for Sema III.

In situ hybridizations

Non-radioactive, digoxigenin (DIG-11-UTP) labeled cRNA probes with either sense or antisense orientation were synthesized by run-off in vitro transcription using T3 and T7 RNA polymerases (Boehringer Mannheim). Probes were generated from three different cDNA templates, rat semaIII cDNA (entire coding region), the extracellular domain of neuropilin (nucleotides 181-2755 of the coding sequence), and a 2.5 kb fragment of neuropilin-2 (downstream of nucleotide 1866). Cryosections (20 μ m) of E14.5 rat embryos (plug day was E1) were cut at -15°C in a Reichert-Jung cryostat and processed for in situ hybridization essentially as described (Giger et al., 1996).

Explant Cocultures and inhibition of Sema III activity

E14 DRG and Sema III expressing COS cells were cocultured for 40hrs as described (Messersmith et al., 1995), except that the culture media was 25% F12 media, 69% OPT-MEM media, 0.04 M glucose, 2mM glutamine, 0.5% heat inactivated fetal calf serum, and NGF (15ng/ml) (purified as described (Mobley et al., 1976)). Media was supplemented with either Anti-neuropilin or pre-immune IgG (100mg/ml). DRG explants and H-sema III-expressing COS cell aggregates were placed ~700 μ m apart. For quantification, the region of neurite growth was divided into

four quadrants, as diagrammed in Figure 4C. Neurite outgrowth into the collagen gel was measured from the outer border of each DRG to the perimeter of the bulk of neurites as described (Messersmith et al., 1995). Fixed cocultures were visualized under phase contrast optics on a Zeiss Axiovert 100 inverted microscope and scored blindly by three independent observers. DRG explants with less than 200mm outgrowth on the distal side were not scored. Statistical analysis was performed using a Student's T-test.

EXAMPLE 4

Neuropilin is the first member of the neuropilin gene family

The great diversity within the semaphorin family of proteins, both with respect to primary amino acid sequence and tissue distribution, led us to investigate the possibility that neuropilin defines a family of conserved semaphorin-binding proteins. A search of the dbEST database identified several human expressed sequence tags that encode proteins either identical to or related to neuropilin. Sequence information from one of these sequence tags was used for the amplification from E14 rat spinal cord/DRG cDNA of a 400 base pair PCR product that was found to encode a portion of a neuropilin-related gene (referred to below as neuropilin-2). This amplification product was used to screen an E14 rat spinal cord/DRG cDNA library. Several cDNAs containing the neuropilin-2 open reading frame were isolated, one

of which was sequenced over the entire neuropilin-2 open reading frame (ORF) (see Experimental Procedures).

Conceptual translation of the neuropilin-2 ORF revealed that it encodes a protein that has the same overall extracellular and intracellular organization as neuropilin (Fig. 5A, B). Like neuropilin (Kawakami et al., 1995; Takagi et al., 1991), neuropilin-2 has (N-terminal to C-terminal) a signal sequence, an a1/a2 domain similar to the noncatalytic regions of the complement components C1r and C1s (CUB domain; (Bork and Beckman, 1993)), a b1/b2 domain similar to the C1 and C2 domains of coagulation factors V and VIII, a c region that contains a MAM domain, a transmembrane domain, and a short cytoplasmic domain unique to neuropilins. The length and spacing of these domains in neuropilin and neuropilin-2 are very similar. Neuropilin and neuropilin-2 share 44% amino acid identity over their entire length, however different domains have different degrees of conservation. For example, the a1/a2 and b1/b2 domains are 55 and 44% identical, respectively, whereas the MAM portions of domain c are only 37% identical. Further, the putative transmembrane domains are 71% identical, and the cytoplasmic domains are 53% identical and of the same length. These features clearly show that neuropilin and neuropilin-2 are members of a gene family encoding related proteins, and have implications for their distinct roles in semaphorin signaling.

Neuropilin-2 identification and molecular analysis

A search of the dbEST data base of human expressed sequence tags identified two overlapping clones, GenBank accession numbers AA057388 and AA057680, that displayed sequence similarity to the 3' end of the neuropilin ORF. Degenerate 5' (TTC/TGAA/GGGIGAA/GATA/C/TGGNAAA/GGG (SEQ ID NO: 5) ; corresponding to the amino acid residues FEGEIGKG (S E Q I D N O : 7)) a n d 3' (NAGT/CTCG/AAAG/ATTG/AATG/ATTT/CTC (SEQ ID NO: 6); corresponding to amino acid residues ENYNFEL(SEQ ID NO: 8)) oligonucleotides were used for PCR-amplification, using 10ng of E14 rat spinal cord/DRG cDNA and employing 45 amplification cycles (96oC 1 min.; 50oC 1 min.; 72oC 1 min.). Amplification products were cloned into pCRII (Invitrogen) and sequenced. One 400 base pair (bp) amplification product encoded a neuropilin-related sequence and was used to screen a rat E14 DRG/spinal cord Lambda Zap II (Stratagene) cDNA library. Several positive clones were isolated, and one 6 kb clone was found to contain the entire neuropilin-2 ORF. 3371 bp of this clone, including the neuropilin ORF, were sequenced on both strands. Alignment of neuropilin and neuropilin-2 sequences was performed using Gene Works (Intelligenetics).

EXAMPLE 5

Neuropilin and neuropilin-2 are expressed in distinct populations of neurons in the developing rat spinal cord

The existence of neuropilin-2 is consistent with the hypothesis that there are multiple, structurally-related semaphorin receptors. Because neuropilin is present in discrete populations of neurons (Kawakami et al., 1995), and because individual semaphorins have distinct neuronal expression patterns (Adams et al., 1996; Giger et al., 1996; Luo et al., 1995; Puschel et al., 1995; Shepherd et al., 1996; Wright et al., 1995; Zhou et al., 1997), we compared the patterns of expression of neuropilin and neuropilin-2 by in situ hybridization. Cross-sections of E14.5 rat embryos stained for the presence of neuropilin mRNA displayed discrete labeling in the spinal cord and a subset of DRG neurons (Fig. 6B), consistent with previous observations of neuropilin expression (Kawakami et al., 1995). For comparison, sema III spinal cord expression is shown in Figure 6A. In the ventral spinal cord, strong neuropilin expression was observed in motor pools and in a thin stripe of cells in the intermediolateral column. Weaker neuropilin expression was detected in the dorsal horn. neuropilin expression was not seen in the spinal neuroepithelium. In contrast, a very different expression pattern was observed for neuropilin-2 (Fig. 6C). Unlike neuropilin, which is strongly expressed in DRG, neuropilin-2 expression was not detected in neurons within the

DRG. Moreover, staining in the spinal cord was largely confined to the ventral horn, the intermediate grey, and a thin dorsally extending stripe of cells at the border of the neuroepithelium. Robust neuropilin-2 expression was seen in the lateral motor pools of the ventral cord and lateral part of the basal plate neuroepithelium. The roof plate and the floor plate also showed moderate neuropilin-2 expression. Examination of other CNS structures revealed that neuropilin and neuropilin-2 are expressed in overlapping, but distinct, populations of neurons. For example, expression of neuropilin, but not neuropilin-2, was detected in the trigeminal ganglion (Fig. 4, data not shown). However, expression of neuropilin-2, but not neuropilin, was observed in the accessory olfactory bulb (data not shown). Lastly, as has been observed for neuropilin, neuropilin-2 expression was not restricted to the nervous system. Strong non-neuronal expression of neuropilin-2 was detected in several tissues, including the mesenchymal tissue lining the ribs (data not shown). Together, these results demonstrate that neuropilin and neuropilin-2 are expressed in overlapping yet distinct populations of neurons in the CNS, and that both genes are expressed in neuronal as well as non-neuronal cells.

References (each of which is expressly incorporated by reference herin)

Ackerman, S. L., Kozak, L. P., Przyborski, S. A., Rund, L. A., Boyer, B. B., and Knowles, B. B. (1997). The mouse rostral cerebellar malformation gene encodes an UNC-5-like protein. *Nature* 386, 838-842.

Adams, R. H., Betz, H., and Puschel, A. W. (1996). A novel class of murine semaphorins with homology to thrombospondin is differentially expressed during early embryogenesis. *Mech. Dev.* 57, 33-45.

Beckmann, G., and Bork, P. (1993). An adhesive domain detected in functionally diverse receptors. *Trends Biochem. Sci.* 18, 40-41.

Behar, O., Golden, J. A., Mashimo, H., Schoen, F. J., and Fishman, M. C. (1996). Semaphorin III is needed for normal patterning and growth of nerves, bones, and heart. *Nature* 383, 525-528.

Bork, P., and Beckman, G. (1993). The CUB domain: A widespread module in developmentally regulated proteins. *J. Mol. Biol.* 231, 539-545.

Cheng, H.-J., and Flanagan, J. G. (1994). Identification and cloning of ELF-1, a developmentally expressed ligand for the Mek4 and Sek receptor tyrosine kinases. *Cell* 79, 157-168.

Fan, J., and Raper, J. A. (1995). Localized collapsing cues can steer growth cones without inducing their full collapse. *Neuron* 14, 263-274.

Fan, J., Mansfield, S. G., Redman, T., Phillip, R., Gordon-Weeks, P. R., and Raper, J. A. (1993). The organization of F-actin and

microtubules in growth cones exposed to a brain-derived collapsing factor. *J. Cell Biol.* 121, 867-878.

Fazeli, A., Dickinson, S. L., Herminston, M. L., Tighe, R. V., Steen, R. G., Small, C. G., Stoeckli, E. T., Keino-Masu, K., Masu, M., Rayburn, H., Simons, J., Bronson, R. T., Gordon, J. I., Tessier-Lavigne, M., and Weinberg, R. A. (1997). Phenotype of mice lacking functional Deleted in colorectal cancer (Dcc) gene. *Nature* 386, 796-804.

Fitzgerald, M., Kwiat, G. C., Middleton, J., and Pini, A. (1993). Ventral spinal chord inhibition of neurite outgrowth from embryonic rat dorsal root ganglia. *Development* 117, 1377-1384.

Flanagan, J. G., and Leder, P. (1990). The kit ligand: A cell surface molecule altered in Steel mutant fibroblasts. *Cell* 63, 185-194.

Giger, R. J., Wolfer, D. P., De Wit, G. M. J., and Verhaagen, J. (1996). Anatomy of rat semaphorin III/collapsin-1 mRNA expression and relationship to developing nerve tracts during neuroembryogenesis. *J. Comp. Neurol.* 375, 378-392.

Ginty, D. D., Bonni, A., and Greenberg, M. E. (1994). Nerve growth factor activates a Ras- dependent protein kinase that stimulates c-fos transcription via phosphorylation of CREB. *Cell* 77, 713-725.

Goshima, Y., Nakamura, F., Strittmatter, P., and Strittmatter, S. M. (1995). Collapsin-induced growth cone collapse mediated by an intracellular protein related to UNC-33. *Nature* 376, 509- 514.

He, Z., and Tessier-Lavigne, M. (1997). Molecular basis of axonal chemorepulsion: Neuropilin is a semaphorin/collapsin receptor. *Cell*, this issue.

Hirata, T., Takagi, S., and Fujisawa, H. (1993). The membrane protein A5, a putative neuronal recognition molecule, promotes neurite outgrowth. *Neurosci. Res.* 17, 159-169.

Ip, N. Y., and Yancopoulos, G. D. (1996). The neurotrophins and CNTF: Two families of collaborative neurotrophic factors. *Ann. Rev. Neurosci.* 19, 491-515.

Ivains, J. K., Raper, J. A., and Pittman, R. N. (1991). Intracellular calcium levels do not change during contact-mediated collapse of chick DRG growth cone structure. *J. Neurosci.* 11, 1597- 1608.

Kawakami, A., Kitsukawa, T., Takagi, S., and Fujisawa, H. (1995). Developmentally regulated expression of a cell surface protein, neuropilin, in mouse nervous system. *J. Neurobiol.* 29, 1-17.

Keynes, R., and Cook, G. M. W. (1995). Axon guidance molecules. *Cell* 83, 161-169.

Kindt, R. M., and Lander, A. D. (1995). Pertussis toxin specifically inhibits growth cone guidance by a mechanism independent of direct G protein inactivation. *Neuron* 15, 79-88.

Kitsukawa, T., Shimono, A., Kawakami, A., Kondoh, H., and Fujisawa, H. (1995). Overexpression of a membrane protein, neuropilin, in chimeric mice causes anomalies in the cardiovascular system, nervous system and limbs. *Development* 121, 4309-4318.

- Kolodkin, A. L. (1996). Semaphorins: mediators of repulsive growth cone guidance. *Trends Cell Biol.* 6, 15-22.
- Kolodkin, A. L., Matthes, D., and Goodman, C. S. (1993). The semaphorin genes encode a family of transmembrane and secreted growth cone guidance molecules. *Cell* 75, 1389-1399.
- Kolodkin, A. L., Matthes, D., O'Connor, T., Patel, N. H., Admon, A., Bentley, D., and Goodman, C. S. (1992). Fasciclin IV: Sequence, expression, and function during growth cone guidance in the grasshopper embryo. *Neuron* 9, 831-835.
- Leonardo, E. D., Hinck, L., Masu, M., Keino-Masu, K., Ackerman, S. L., and Tessier-Lavigne, M. (1997). Vertebrate homologues of *C. elegans* UNC-5 are candidate netrin receptors. *Nature* 386, 833-838.
- Luo, Y., Raible, D., and Raper, J. A. (1993). Collapsin: a protein in brain that induces the collapse and paralysis of neuronal growth cones. *Cell* 75, 217-227.
- Luo, Y., Shepherd, I., Li, J., Renzi, M. J., Chang, S., and Raper, J. (1995). A family of molecules related to collapsin in the embryonic chick nervous system. *Neuron* 14, 1131-1140.
- Matthes, D. J., Sink, H., Kolodkin, A. L., and Goodman, C. S. (1995). Semaphorin II can function as a selective inhibitor of specific synaptic arborizations in *Drosophila*. *Cell* 81, 631-639.
- McIntire, S. L., Garriga, G., White, J., Jacobson, D., and Horvitz, H. R. (1992). Genes necessary for directed axonal elongation or fasciculation in *C. elegans*. *Neuron* 8, 307-322.
- Messersmith, E. K., Leonardo, E. D., Shatz, C. J., Tessier-Lavigne, M., Goodman, C. S., and Kolodkin, A. L. (1995). Semaphorin III can function as a selective chemorepellent

to pattern sensory projections in the spinal cord. *Neuron* 14, 949-959.

Mobley, W. C., Schenker, A., and Shooter, E. M. (1976). Characterization and isolation of proteolytically modified nerve growth factor. *Biochem.* 15, 5543-5551.

Monschau, B., Kremoser, C., Ohta, K., Tanaka, H., Kaneko, T., Yamada, T., Hardwerker, C., Hornberger, M. R., Loschinger, J., Pasquale, E. B., Siever, D. A., Verderame, M. F., Muller, B. K., Bonhoeffer, F., and Drescher, U. (1997). Shared and distinct functions of RAGS and Elf-1 in guiding retinal axons. *EMBO J.* 16, 1258-1267.

Pearson, A., Lux, A., and Krieger, M. (1995). Expression cloning of dSR-CI, a class C macrophage-specific scavenger receptor from *Drosophila melanogaster*. *Proc. Natl. Acad. Sci. USA* 92, 4065-4060.

Puschel, A. W. (1996). The Semaphorins: A family of axonal guidance molecules? *Euro. J. Neurosci* 8, 1317-1321. Puschel, A. W., Adams, R. H., and Betz, H. (1995). Murine semaphorin D/collapsin is a member of a diverse gene family and creates domains inhibitory for axonal extension. *Neuron* 14, 941-948.

Puschel, A. W., Adams, R. H., and Betz, H. (1996). The sensory innervation of the mouse spinal cord may be patterned by differential expression of and differential responsiveness to semaphorins. *Mol. Cell. Neurosci.* 7, 419-431.

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual*, Second Edition (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory).

Satoda, M., Takagi, S., Ohta, K., Hirata, T., and Fujisawa, H. (1995). Differential expression of two cell surface proteins,

neuropilin and plexin, in *Xenopus* olfactory axon subclasses. *J. Neurosci.* 15, 942-955.

Serafini, T., Kennedy, T., Galko, M., Mirzayan, C., Jessell, T., and Tessier-Lavigne, M. (1994). The netrins define a family of axon outgrowth-promoting proteins with homology to *C. Elegans* UNC-6. *Cell* 78, 409-424.

Shepherd, I. T., Luo, Y., Lefcort, F., Reichardt, L. F., and Raper, J. A. (1997). A sensory axon repellent secreted from ventral spinal cord explants is neutralized by antibodies raised against collapsin-1. *Development* 124, 1377-1385.

Shepherd, I. T., Luo, Y., Raper, J. A., and Chang, S. (1996). The distribution of collapsin-1 mRNA in the developing chick nervous system. *Dev. Biol.* 173, 185-199.

Shirasaki, R., Tamada, A., Katsumata, R., and Murakami, F. (1995). Guidance of cerebellofugal axons in the rat embryo: Directed growth toward the floor plate and subsequent elongation along the longitudinal axis. *Neuron* 14, 961-972.

Takagi, S., Hirata, T., Agata, K., Mochii, M., Eguchi, G., and Fujisawa, H. (1991). The A5 antigen, a candidate for the neuronal recognition molecule, has homologies to complement components and coagulation factors. *Neuron* 7, 295-307.

Takagi, S., Kasuya, Y., Shimizu, M., Matsuura, T., Tsuboi, M., Kawakami, A., and Fujisawa, H. (1995). Expression of a cell adhesion molecule, neuropilin, in the developing chick nervous system. *Dev. Biol.* 170, 207-222.

Takagi, S., Tsuji, T., Amagai, T., Takamatsu, T., and Fujisawa, H. (1987). Specific cell surface labels in the visual center of *Xenopus laevis* tadpole identified using monoclonal antibodies. *Dev. Biol.* 122, 90-100.

Tessier-Lavigne, M., and Goodman, C. S. (1996). The molecular biology of axon guidance. *Science* 274, 1123-1133.

Varela-Echavarria, A., Tucker, A., Puschel, A. W., and Guthrie, S. (1997). Motor axon subpopulations respond differentially to the chemorepellents netrin-1 and semaphorin D. *Neuron* 18, 193-207.

Wang, L.-H., and Strittmatter, S. M. (1996). A family of rat CRMP genes is differentially expressed in the nervous system. *J. Neurosci.* 16, 6197-6207.

Wright, D. E., White, F. A., Gerfen, R. W., Silos-Santiago, I., and Snider, W. D. (1995). The guidance molecule semaphorin III is expressed in regions of spinal cord and periphery avoided by growing sensory axons. *J. Comp. Neurol.* 361, 321-333.

Zhou, L., White, F. A., Lentz, S. I., Wright, D. E., Fisher, D. A., and Snider, W. D. (1997). Cloning and expression of a novel murine semaphorin with structural similarity to insect semaphorin I. *Mol. Cell. Neurosci.* 9, 26-41.

CLAIMS

1. A method of monitoring the interaction of a semaphorin and a neuropilin, comprising the steps of:

contacting a first protein which comprises an extracellular domain of a neuropilin with a second protein which comprises an extracellular domain of a semaphorin under conditions where the extracellular domain of the neuropilin binds to the extracellular domain of the semaphorin;

determining the binding of the first protein to the second protein or second protein to the first protein.

2. The method of claim 1 wherein the step of determining comprises:

contacting the first and second proteins with an antibody which specifically binds to the first protein or the second protein to form an immunoprecipitate;

determining the presence of the first protein in the immunoprecipitate if an antibody which specifically binds to the second protein was used, and determining the presence of the second protein in the immunoprecipitate if an antibody which specifically binds to the first protein was used.

3. The method of claim 1 wherein the first or second protein is bound to a solid support.

4. The method of claim 1 wherein the first protein lacks a transmembrane and an intracellular domain of the neuropilin.

5. The method of claim 1 wherein one of the two proteins is myc-tagged and an anti-myc antibody is used.

6. The method of claim 1 wherein one of the two proteins is a fusion protein comprising an enzyme which produces a readily detectable product in the presence of substrate.

7. The method of claim 6 wherein the enzyme is alkaline phosphatase.

8. The method of claim 1 wherein a test compound is added to the first and second proteins to determine its effect on binding of the first protein to the second protein.

9. The method of claim 1 wherein the neuropilin is neuropilin-1.

10. The method of claim 1 wherein the neuropilin is neuropilin-2.

11. The method of claim 1 wherein the semaphorin is semaphorin

III.

12. An isolated and purified subgenomic nucleic acid molecule which encodes a mammalian neuropilin-2, having at least 90% sequence identity to SEQ ID NO: 1.

13. The subgenomic nucleic acid molecule of claim 12 which encodes the amino acid sequence of SEQ ID NO: 2.

14. The subgenomic nucleic acid molecule of claim 12 which comprises the nucleotide sequence of SEQ ID NO: 1.

15. The subgenomic nucleic acid molecule of claim 12 which is intron-free.

16. The subgenomic nucleic acid molecule of claim 12 which shares at least 92% nucleic acid identity with the nucleotide sequence of SEQ ID NO: 1.

17. The subgenomic nucleic acid molecule of claim 12 which encodes a protein which shares greater than 90% amino acid identity with the amino acid sequence of SEQ ID NO: 2.

18. A method of identifying axon guidance cues, comprising the steps of:

contacting a detectably labeled mammalian neuropilin protein or semaphorin-binding portion thereof, with a mixture of proteins secreted by neuronal cells;

removing proteins of the mixture which do not bind to the detectably labeled mammalian neuropilin; wherein a protein of the mixture which binds to the detectably labeled mammalian neuropilin or said portion is identified as a candidate axon guidance cue.

19. The method of claim 18 wherein the neuropilin protein is neuropilin-1.

20. The method of claim 18 wherein the neuropilin protein is neuropilin-2.

21. A method for monitoring the interaction between a semaphorin and a neuropilin, comprising the steps of:

contacting a protein comprising a semaphorin sema or Ig basic domain with cells which express a polypeptide comprising an extracellular domain of a neuropilin;

detecting the protein comprising the semaphorin sema or Ig basic domain which binds to the cells.

22. The method of claim 21 wherein the protein comprising the semaphorin domain is a fusion protein.

23. The method of claim 21 wherein the cells are transfected with an expression construct encoding a polypeptide comprising a neuropilin extracellular domain.

24. The method of claim 21 further comprising the step of:
prior to the step of contacting, determining that the cells express the extracellular domain of neuropilin.

25. The method of claim 21 wherein the semaphorin is semaphorin III.

26. The method of claim 21 wherein the polypeptide is neuropilin-1.

27. The method of claim 21 wherein the polypeptide is neuropilin-2.

28. The method of claim 22 wherein the fusion protein further comprises alkaline phosphatase.

29. The method of claim 28 wherein a substrate for alkaline phosphatase is added which forms a colored product.

30. The method of claim 23 wherein the cells are COS cells.

31. A polypeptide portion of neuropilin useful for antagonizing the interaction between a semaphorin and a neuropilin, comprising an extracellular domain of a neuropilin.

32. The polypeptide of claim 31 wherein the neuropilin is neuropilin-1.

33. The polypeptide of claim 31 wherein the neuropilin is neuropilin-2.

34. The polypeptide of claim 31 wherein the extracellular domain is a B domain of neuropilin.

35. The polypeptide of claim 34 wherein the B domain is of neuropilin-1.

36. The polypeptide of claim 34 wherein the B domain is of neuropilin-2.

37. The polypeptide of claim 31 wherein the extracellular domain is an A domain.

38. The polypeptide of claim 31 wherein the extracellular domain is a MAM domain.

39. The polypeptide of claim 37 wherein the A domain is of neuropilin-1.

40. The polypeptide of claim 37 wherein the A domain is of neuropilin-2.

41. An isolated and purified protein which is neuropilin-2 as shown in SEQ ID NO: 2.

42. A method of monitoring the interaction between a semaphorin and a neuropilin comprising the steps of:

coculturing in a matrix (a) embryonic nerve cells with (b) cells which have been transfected with an expression construct encoding a semaphorin and which express the semaphorin;

adding to the cells a compound to be tested as an inhibitor of binding of the semaphorin and the neuropilin;

determining the inhibition of embryonic nerve cell axon outgrowth adjacent to the cells which express the semaphorin in the presence and absence of the test compound.

43. The method of claim 42 wherein the semaphorin is semaphorin III.

44. The method of claim 42 wherein the cells which express a semaphorin are COS cells.

45. The method of claim 42 wherein the test compound is an anti-neuropilin antibody.

46. The method of claim 42 wherein the test compound is a polypeptide comprising a B domain of a neuropilin.

47. The method of claim 42 wherein the test compound is a polypeptide comprising an extracellular domain of a neuropilin.

48. A method of monitoring the interaction between a semaphorin and a neuropilin comprising the steps of:

culturing embryonic nerve cells under conditions in which they display growth cones;

contacting the embryonic nerve cells with a semaphorin and a compound to be tested for inhibiting the interaction of the semaphorin and a neuropilin;

observing the effect of the inhibitor on the collapse of the growth cones.

49. The method of claim 48 wherein the semaphorin is semaphorin III.

50. The method of claim 48 wherein the test compound is an anti-neuropilin antibody.

51. An antibody preparation which specifically binds to a neuropilin protein, with the proviso that the neuropilin protein is not neuropilin-1.

52. An antibody preparation which specifically binds to an extracellular domain of a neuropilin protein and does not specifically bind to the intracellular domain of the neuropilin protein.

53. The antibody of claim 52 which binds to a B domain of the neuropilin protein.

54. The antibody of claim 52 which inhibits growth cone collapse induced by a semaphorin.

55. The antibody of claim 54 wherein the semaphorin is semaphorin III.

56. The antibody of claim 52 wherein the neuropilin protein is neuropilin-2.

57. An isolated and purified mammalian neuropilin protein which comprises a signal sequence, two complement binding domains, two

coagulation factor domains, a MAM domain, a transmembrane domain and a cytoplasmic domain, with the proviso that the neuropilin protein is not neuropilin-1.

58. The polypeptide of claim 31 which is covalently bound to a second polypeptide which has a biological function which neuropilin does not possess.

59. The polypeptide of claim 31 which is bound to a solid support.

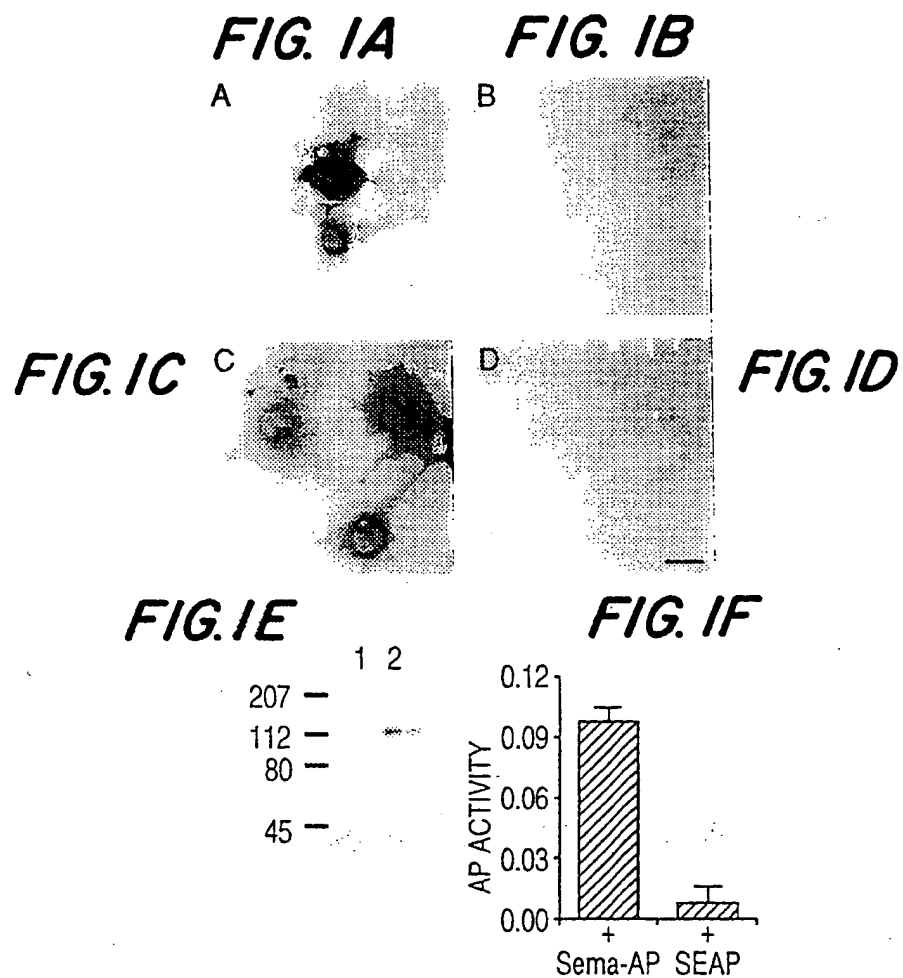


FIG. 2A FIG. 2C FIG. 2E

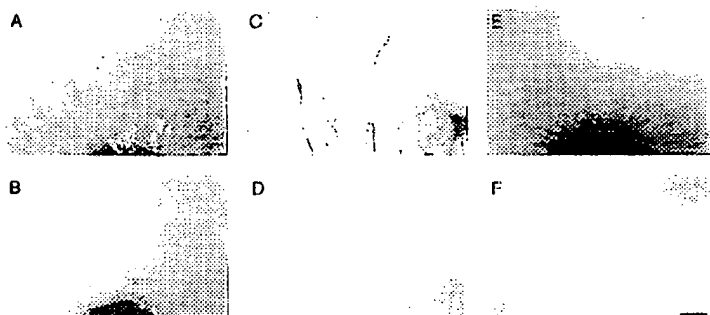
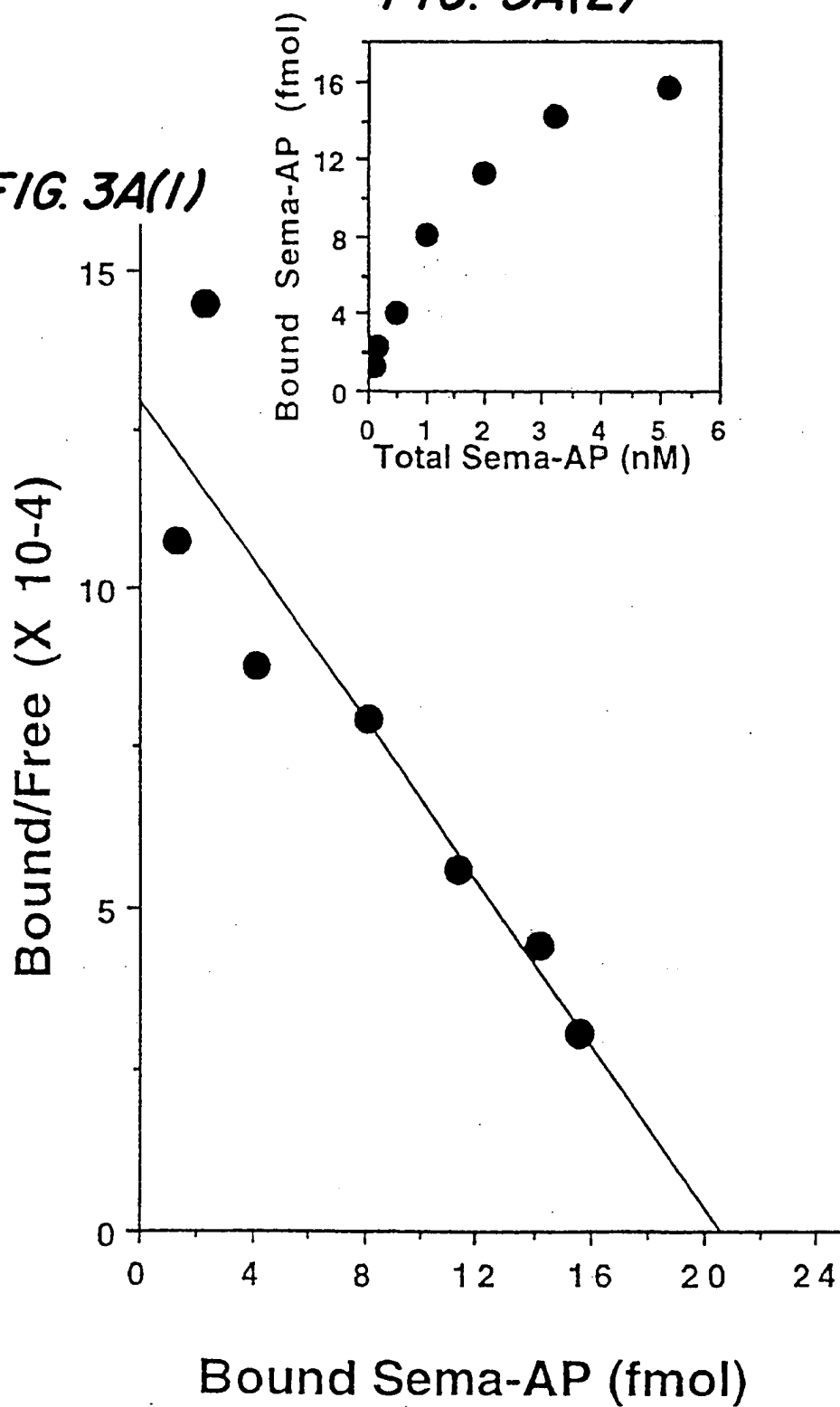


FIG. 2B FIG. 2D FIG. 2F

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FIG. 3A(2)**FIG. 3A(1)**

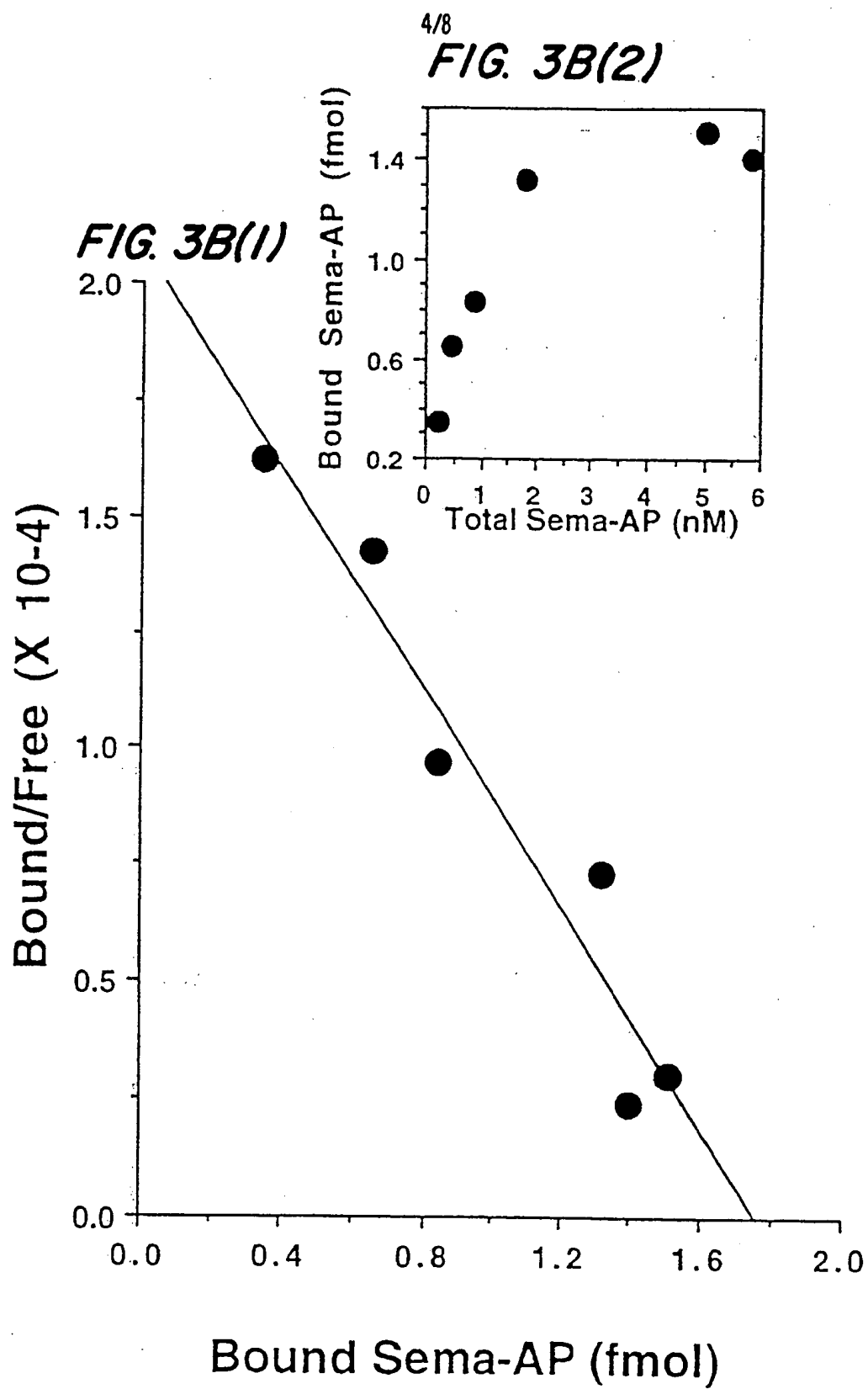


FIG. 4A

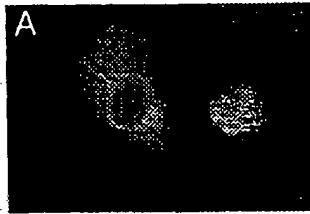


FIG. 4B

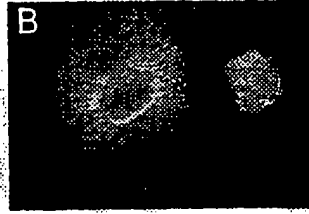


FIG. 4C

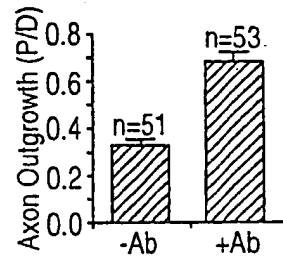
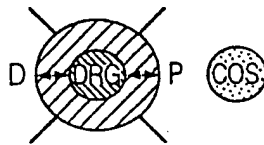


FIG. 4D

FIG. 4E

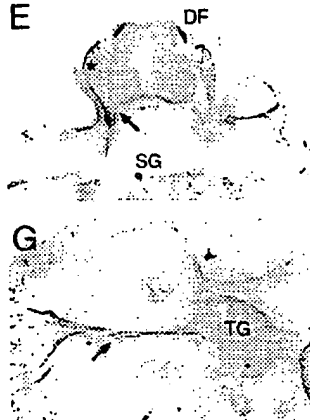


FIG. 4F

FIG. 4G



FIG. 4H



FIG. 5A

neuropilin-2	MDMFPITNIFLALYFSGHKVRSQDDPPCGRLNSKIDAGYITSPGYPODYP SHONCEWVYAPENKIVLNFNPH	75
neuropilin	MER-GLPLLCATLALALAGAFRSDDKCGGTIKIENPGYLTSPGYPHSYHPSEKCEWLTQAPEPYQRIIMINFNPH	74
neuropilin-2	FEIEKHDCKYDFEIRDGDSEADLLGKHCNTAPPTIISSGSVLYIKFISDYARQGAGFSURYEIFKISSEDCS	150
neuropilin	FDLEDRCCKYDVENIDGENEGGRLAGKFCGKIAPSPVSSGPFILKFNVDYETHGAGFSIRYEIFKRQPELCS	148
neuropilin-2	KNFTSPNGITTESPGFPEKYPHLLDCTFTILAKPRMETTLOFLTDLHDPLOVGEQDQYDMLDIWDGIPVGPIL	225
neuropilin	QNYTAPITGMKSPGFPEKYPNSLECTYIIFAPKMSEIILEFESFLEDDSNPPGGVFORIDREIWDGFPEVGP	223
neuropilin-2	IGKYCGIKTPSKLRSSIGILSLTFHTDMAKDGFSARYLVHQEPPENFQNAPELGMESGRIVNEOTISASTFS	300
neuropilin	IGRYCGKTPGRIRSSGILSHVFTDSATAKEGFSANYSVLOSSISEDFKMEALGMESGEIHSOITASSOY-	297
neuropilin-2	DGRMTPOQSRLHGDNGWTPNVDNKEYLOVDRFETMLTALATOGATSRETDKGYVVKSYKLEVSINGEDMNVY	375
neuropilin	GTNMSVERSLNYPENGWTPGDSYREWIDVDLGLRFVTAVGTGAI SKE TKKYYVKIYRVDISSNGEDMITL	372
neuropilin-2	RHGKNHKVFOANNATELVLNKLHTPLITRFIRIRPOTMHLGIALRLFLFGQVTDAPCSNMLGMSGLIADHDI	450
neuropilin	KEGNKALIFQGNTPITDMVGVFPKPLITRFIRIRKPSMETGTISMRFVYVGKLTIDYPCSGMLGMSGLISDSOI	447
neuropilin-2	SASSTREYLWSPSAARLVSSRSGWFPNRPQAOPGEWLOVDLGTPIKMGVITOGARGGDSITAMEARAFVRKFK	525
neuropilin	TASNOGDRNMPENIRLVISRIQWALPPSPHPYINNEWLOVDLGEKIMRGVITOGGKHREN-----KVFMRKFK	516
neuropilin-2	VSYSLNGKOWEYIDPRTQPKLFEGNMHTDPIRRFEPVPAQVIRVYPERWSPAGTGNRLVLCDD--WTDCK	598
neuropilin	IAYSNGSDWKIMDDSKRKAKSEGNMHTDPELRAFDPLSTRFIRIRYPERATHSGGLRMELLGCEVEVPTAG	591
neuropilin-2	PTVETLGPTVKSEE-----ITTPYPMDEATE-CGNCSEFDK-DLQPS-GFNQDF-PEETI-CGMNYD	660
neuropilin	PTTPNGNPVDECDDOANCHSGTGDDFQLTGGTIVLATEKPTIIDSTIOSEFTYGFNCEFGWGSKHITFCHMHD	666
neuropilin-2	RAKWLQSTWISSANPNDRTPDQKNTFLKLSDDGGREGQFRLIISPPVHLPRSPVCMEEFYQAMGGHVALDM-VR	734
neuropilin	SHAQLRWRVLTSGTGPIQDHTGQGNFIYSQADENOKQVARLMSPMVYSQSAHCMIFMYHMSGSVGTLRVKLH	741
neuropilin-2	EAROE--SKLWVIREDDGSEWKGRIILPSYDMEYQVFEGMTGKRSGETSIDDIRISTQVPLENCEPIISAF	807
neuropilin	YQKPEFYDQVMVVGHGQDHMKEGRYLLHKSCLKYQVFEGETGKNLGGIAVDDISINNHIPQEDCAKP-TDL	815
neuropilin-2	AVDIPETHGGEGYEDEIDDDYEGDWNHSSSTSGAGSPSSGKEKSWLYTLDPIIITIIAMSSLGVLGAGCAGLL	882
neuropilin	DKKNTETIKID---ETGSTPGYEKGKQKNEISRKPGN-----KTLDPIIITIIAMSSALGVLLGAGCGVVLV	879
neuropilin-2	YQICSYSLSSRSCTTLENYNFELYDGLR-HKVNTHQKCCSEA	925
neuropilin	-CACWHNGMSERNLSALENYNFELVDGVLKQDKLNPQSNYSEA	922

FIG. 5B

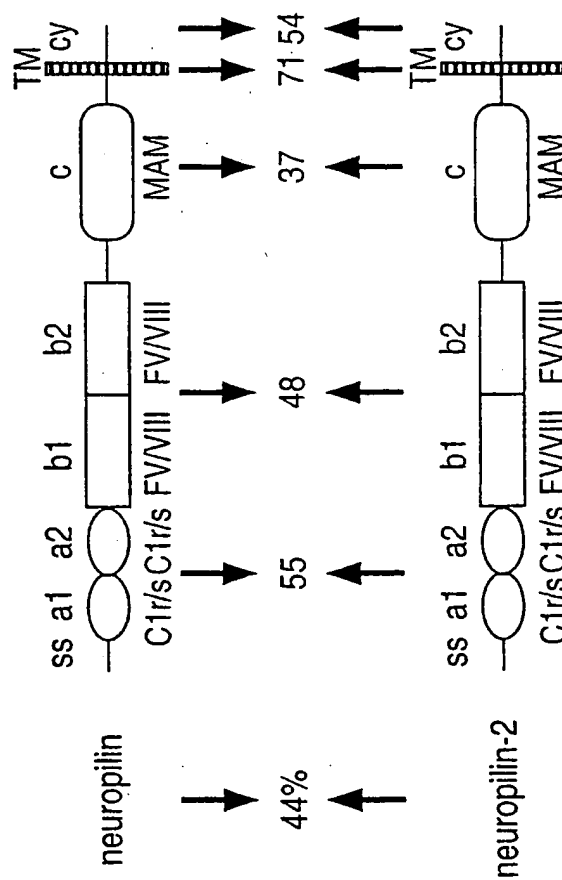


FIG. 6A

FIG. 6B

FIG. 6C



SEQUENCE LISTING

(1) GENERAL INFORMATION

- (i) APPLICANT: Kolodkin, Alex
Ginty, David
- (ii) TITLE OF THE INVENTION: SEMAPHORIN RECEPTOR
- (iii) NUMBER OF SEQUENCES: 8
- (iv) CORRESPONDENCE ADDRESS:
 - (A) ADDRESSEE: Banner & Witcoff
 - (B) STREET: 1001 G Street, NW
 - (C) CITY: Washington
 - (D) STATE: DC
 - (E) COUNTRY: USA
 - (F) ZIP: 20001
- (v) COMPUTER READABLE FORM:
 - (A) MEDIUM TYPE: Diskette
 - (B) COMPUTER: IBM Compatible
 - (C) OPERATING SYSTEM: DOS
 - (D) SOFTWARE: FastSEQ for Windows Version 2.0
- (vi) CURRENT APPLICATION DATA:
 - (A) APPLICATION NUMBER:
 - (B) FILING DATE: 17-JUL-1998
 - (C) CLASSIFICATION:
- (vii) PRIOR APPLICATION DATA:
 - (A) APPLICATION NUMBER: 60/052,762
 - (B) FILING DATE: 17-JUL-1998
- (viii) ATTORNEY/AGENT INFORMATION:
 - (A) NAME: Kagan, Sarah A
 - (B) REGISTRATION NUMBER: 32141
 - (C) REFERENCE/DOCKET NUMBER: 01107.74973
- (ix) TELECOMMUNICATION INFORMATION:
 - (A) TELEPHONE: 202-508-9100
 - (B) TELEFAX: 202-508-9299
 - (C) TELEX:

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 3371 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

TAGATCCCCG	GGCTGCAGGA	ATTCTGCGGC	CGCAGACCAT	ACACGCGTTT	GGGTTTGAAG	60
AGGAAACTGG	TCTCCGCTTC	CCCAGCTTGC	TCCCTCTTTG	CTGATTTTCAG	GGGCTATCTC	120
TTAGTGAGGT	GGAGATATTC	CAGCAAGAAT	AAAGGTGAAG	GCAGACGGAC	CTCCAGGACG	180
CAGGAGGAAA	ACGCTGATCA	TTAGAGACCT	TTGCAGAAGA	CACCACAAGG	AAGAAAATTA	240
GAGAGGAAAA	ACACAAAGAC	ATTATACGAG	ATCCCACCAA	CCTAGCCCTG	GAGAGAGCCT	300
CTCTGTCAAA	AATGGATATG	TTTCTCTCA	CCTGGATTTT	CTTAGCTCTG	TACTTTTCGG	360
GACACAAAGT	GAGAAGCCAG	CAAGATCCGC	CCTGCGGAGG	TCGGCTGAAT	TCCAAAGATG	420
CTGGCTATAT	CAC TTCCCCA	GGTTACCCCC	AGGACTATCC	CTCTCACCAG	AACTGTGAGT	480
GGGTTGTCTA	TGCCCCCGAA	CCCAACCAGA	AGATTGTCCT	CAACTTCAAC	CCTCACTTTG	540
AAATCGAGAA	GCATGACTGC	AAGTATGACT	TCATTGAGAT	TCGGGATGGA	GACAGTGAGT	600
CAGCTGACCT	CCTGGGCAAG	CAC TGTGGGA	ACATTGCCCC	TCCCACCATC	ATCTCTTCCG	660
GCTCCGTGTT	ATATATCAAG	TTCACATCAG	ACTACGCCCC	GCAGGGGGCA	GGTTTCTCCC	720
TACGCTATGA	GATCTTCAAA	ACAGGCTCTG	AAGATTGTTT	CAAGAACTTT	ACAAGCCCCA	780
ATGGGACCAT	TGAATCTCCA	GGGTTTCCAG	AGAAATATCC	ACACAATCTG	GACTGTACCT	840
TCACCATCCT	GGCCAAACCC	AGGATGGAGA	TCATCCTACA	GTTCTGACC	TTTGACCTGG	900
AGCATGACCC	TCTACAAGTG	GGGGAAGGAG	ACTGTAAATA	TGACTGGCTG	GACATCTGGG	960
ATGGCATTCC	ACATGTTGGG	CCTCTGATTG	GCAAGTACTG	TGGGACGAAA	ACACCTCCA	1020
AACTCCGCTC	GTCCACAGGG	ATCCTCTCCC	TGACCTTTCA	CACCGACATG	GCCGTGGCCA	1080
AGGATGGCTT	CTCAGCACGT	TACTATTTGG	TCCACCAAGA	ACCACCTGAG	AACTTTCAGT	1140
GCAATGCCCC	TCTGGGAATG	GAGTCTGGCC	GGATTGCTAA	TGAACAGATC	AGTGCCTCAT	1200
CCACCTTCTC	TGATGGGAGG	TGGACTCCTC	AACAGAGCAG	GCTCCATGGT	GATGACAATG	1260
GCTGGACACC	CAACGTGGAT	TCCAACAAGG	AGTATCTCCA	GGTGGACCTG	CGCTTCTTAA	1320
CCATGCTCAC	AGCCATTGCA	ACACAAGGAG	CCATTTCCAG	GGAGACCCAG	AAGGGCTACT	1380
ACGTCAAATC	GTACAAGCTG	GAAGTCAGCA	CAAACGGGGA	AGATTGGATG	GTCTACCGGC	1440
ATGGCAAAAA	CCACAAGGTA	TTCCAGGCTA	ACAATGATGC	CACCGAGTTG	GTTCTGAACA	1500
AGCTGCACAC	GCCGCTGTTG	ACTCGTTTCA	TCAGGATCCG	CCCGCAGACG	TGGCATTGTTG	1560
GCATAGCCCT	TCGACTGGAG	CTCTTTGGTT	GCCGGGTCAC	AGATGCACCC	TGCTCCAACA	1620
TGCTGGGAAT	GCTCTCGGGC	CTCATTGCTG	ATACCCAGAT	CTCTGCCTCC	TCCACCCGAG	1680
AGTACCTCTG	GAGCCCCAGT	GCTGCCCGCC	TGGTTAGCAG	CCGCTCTGGC	TGGTTCCCTC	1740
GGAACCTCTA	AGCCCAGCCA	GGTGAAGAAT	GGCTTCAGGT	GGATCTTGGG	ACACCCAAGA	1800
CGGTGAAAGG	CGTOATCATC	CAGGGGGCCC	GAGGAGGAGA	CAGTATCACT	GCCATGGAAG	1860
CCAGGGCATT	TGTACGCAAG	TTCAAAGTCT	CCTACAGCCT	AAATGGCAAG	GACTGGGAAT	1920
ATATCCAGGA	CCCCAGGACT	CAGCAGCCAA	AGCTGTTTGA	AGGGAACATG	CACTATGACA	1980
CCCCCGACAT	CCGAAGGTTT	GAGCCAGTTC	CAGCACAGTA	CGTGCGGGTA	TACCCAGAGA	2040
GGTGGTCACC	AGCGGGCATC	GGGATGAGGC	TGGAGGTCCT	GGGCTGTGAC	TGGACAGACT	2100
CAAAGCCCAC	AGTGAGACG	CTGGGACCCA	CCGTAAAGAG	TGAAGAGACC	ACCACCCCAT	2160
ATCCCATGGA	TGAGGATGCC	ACGGAGTGTG	GGGAAAAC TG	CAGCTTTGAG	GATGACAAAG	2220
ATTTGCAACT	TCCTTCAGGA	TTCAACTGCA	ACTTTGATTT	TCCTGAAGAG	ACCTGTGGTT	2280
GGATGTACGA	CCGTGCCAAG	TGGCTGCAGA	GTACCTGGAT	CAGCAGTGCC	AACCCTAACG	2340
ACAGAACGTT	TCCAGATGAC	AAGAACTTCC	TGAAACTACA	GAGCGACGGC	GGACGAGAGG	2400
GCCAGTTTGG	GCGGCTCATC	AGCCCACACG	TGCACCTGCC	CCGAAGCCCT	GTGTGCATGG	2460
AGTTCCAATA	CCAAGCCATG	GGCGGCCACG	GGGTGGCACT	GCAGGTGGTT	CGGGAAGCCA	2520
GACAGGAAAG	CRAACTCCTT	TGGGTCATCC	GCGAGGACCA	GGGCAGCGAG	TGGAAGCATG	2580
GACGCATTAT	CCTGCCCAGC	TATGACATGG	AGTATCAGAT	CGTATTCGAG	GGAGTGATCG	2640

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GGAAAGGGCG ATCCGGAGAG ATTTCCATCG ACCACATTCG GATAAGCACC GATGTCCCAC 2700
TGGAGAAGCTG CATGGAACCC ATCTCGGCTT TTGCAGTGGA CATCCCAGAA ATCCATGGGG 2760
GAGAGGGGCTA TGAAGATGAG ATTGATGATG ACTATGAAGG AGATTGGAAC AACTCTTCCT 2820
CTACCTCAGG GGCTGGTAGT CCTCATCTG GCAAAGAAAA GAGCTGGCTG TACACACTGG 2880
ACCCCATCCT GATCACCATC ATTGCCATGA GCTCGCTGGG TGTCTGCTG GGGGCCACCT 2940
GTGCGGGCCT CCTCTCTAC TGCACCTGCT CCTACTCTGG CCTGAGTTCG AGAAGCTGCA 3000
CCACACTGGA GAACTACAAC TTTGAGCTCT ACGACGGCCT CAAGCACAAG GTCAGATCA 3060
ATCACCAGAA GTGCTGCTCG GAGGCATGAC CGATTGTGTC TGAATCGCTT CTGGCGTTTC 3120
ATTCCAGCGA GAGGGGCTAG GGAAGATTAC TTTTTTTTC CTTTGGAAC TGAATGCCAT 3180
AATCTGGATC AAACCGATCC AGAATACTGA AGGTATGGAC AGAACAGACA GGCCAGTCTA 3240
GGAGAAAGGA AGATGCAGCC GTGAAGGGGA TCATTGCCCA CAGAGGACAG TGGTGGTCAA 3300
GTTAATGCAG GAACCGGGCC CGTGTCTCT GCGGGACAC AGACAGGAGC GCATCTCCTC 3360
GGAGTCAACA G 3371

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(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 925 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```

Met Asp Met Phe Pro Leu Thr Trp Ile Phe Leu Ala Leu Tyr Phe Ser
 1             5             10             15
Gly His Lys Val Arg Ser Gln Gln Asp Pro Pro Cys Gly Gly Arg Leu
 20             25             30
Asn Ser Lys Asp Ala Gly Tyr Ile Thr Ser Pro Gly Tyr Pro Gln Asp
 35             40             45
Tyr Pro Ser His Gln Asn Cys Glu Trp Val Val Tyr Ala Pro Glu Pro
 50             55             60
Asn Gln Lys Ile Val Leu Asn Phe Asn Pro His Phe Glu Ile Glu Lys
 65             70             75             80
His Asp Cys Lys Tyr Asp Phe Ile Glu Ile Arg Asp Gly Asp Ser Glu
 85             90             95
Ser Ala Asp Leu Leu Gly Lys His Cys Gly Asn Ile Ala Pro Pro Thr
100            105            110
Ile Ile Ser Ser Gly Ser Val Leu Tyr Ile Lys Phe Thr Ser Asp Tyr
115            120            125
Ala Arg Gln Gly Ala Gly Phe Ser Leu Arg Tyr Glu Ile Phe Lys Thr
130            135            140
Gly Ser Glu Asp Cys Ser Lys Asn Phe Thr Ser Pro Asn Gly Thr Ile
145            150            155            160
Glu Ser Pro Gly Phe Pro Glu Lys Tyr Pro His Asn Leu Asp Cys Thr
165            170            175
Phe Thr Ile Leu Ala Lys Pro Arg Met Glu Ile Ile Leu Gln Phe Leu
180            185            190
Thr Phe Asp Leu Glu His Asp Pro Leu Gln Val Gly Glu Gly Asp Cys
195            200            205
Lys Tyr Asp Trp Leu Asp Ile Trp Asp Gly Ile Pro His Val Gly Pro
210            215            220

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Leu Ile Gly Lys Tyr Cys Gly Thr Lys Thr Pro Ser Lys Leu Arg Ser
 225 230 235 240
 Ser Thr Gly Ile Leu Ser Leu Thr Phe His Thr Asp Met Ala Val Ala
 245 250 255
 Lys Asp Gly Phe Ser Ala Arg Tyr Tyr Leu Val His Gln Glu Pro Pro
 260 265 270
 Glu Asn Phe Gln Cys Asn Ala Pro Leu Gly Met Glu Ser Gly Arg Ile
 275 280 285
 Val Asn Glu Gln Ile Ser Ala Ser Ser Thr Phe Ser Asp Gly Arg Trp
 290 295 300
 Thr Pro Gln Gln Ser Arg Leu His Gly Asp Asp Asn Gly Trp Thr Pro
 305 310 315 320
 Asn Val Asp Ser Asn Lys Glu Tyr Leu Gln Val Asp Leu Arg Phe Leu
 325 330 335
 Thr Met Leu Thr Ala Ile Ala Thr Gln Gly Ala Ile Ser Arg Glu Thr
 340 345 350
 Gln Lys Gly Tyr Tyr Val Lys Ser Tyr Lys Leu Glu Val Ser Thr Asn
 355 360 365
 Gly Glu Asp Trp Met Val Tyr Arg His Gly Lys Asn His Lys Val Phe
 370 375 380
 Gln Ala Asn Asn Asp Ala Thr Glu Leu Val Leu Asn Lys Leu His Thr
 385 390 395 400
 Pro Leu Leu Thr Arg Phe Ile Arg Ile Arg Pro Gln Thr Trp His Leu
 405 410 415
 Gly Ile Ala Leu Arg Leu Glu Leu Phe Gly Cys Arg Val Thr Asp Ala
 420 425 430
 Pro Cys Ser Asn Met Leu Gly Met Leu Ser Gly Leu Ile Ala Asp Thr
 435 440 445
 Gln Ile Ser Ala Ser Ser Thr Arg Glu Tyr Leu Trp Ser Pro Ser Ala
 450 455 460
 Ala Arg Leu Val Ser Ser Arg Ser Gly Trp Phe Pro Arg Asn Pro Gln
 465 470 475 480
 Ala Gln Pro Gly Glu Glu Trp Leu Gln Val Asp Leu Gly Thr Pro Lys
 485 490 495
 Thr Val Lys Gly Val Ile Ile Gln Gly Ala Arg Gly Gly Asp Ser Ile
 500 505 510
 Thr Ala Met Glu Ala Arg Ala Phe Val Arg Lys Phe Lys Val Ser Tyr
 515 520 525
 Ser Leu Asn Gly Lys Asp Trp Glu Tyr Ile Gln Asp Pro Arg Thr Gln
 530 535 540
 Gln Pro Lys Leu Phe Glu Gly Asn Met His Tyr Asp Thr Pro Asp Ile
 545 550 555 560
 Arg Arg Phe Glu Pro Val Pro Ala Gln Tyr Val Arg Val Tyr Pro Glu
 565 570 575
 Arg Trp Ser Pro Ala Gly Ile Gly Met Arg Leu Glu Val Leu Gly Cys
 580 585 590
 Asp Trp Thr Asp Ser Lys Pro Thr Val Glu Thr Leu Gly Pro Thr Val
 595 600 605
 Lys Ser Glu Glu Thr Thr Thr Pro Tyr Pro Met Asp Glu Asp Ala Thr
 610 615 620
 Glu Cys Gly Glu Asn Cys Ser Phe Glu Asp Asp Lys Asp Leu Gln Leu
 625 630 635 640
 Pro Ser Gly Phe Asn Cys Asn Phe Asp Phe Pro Glu Glu Thr Cys Gly

(2) INFORMATION FOR SEQ ID NO:3:

(A) LENGTH: 3471 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

GGCACGAGGA	CCGGCTGAGG	ATTTTATGGT	TCTTAAGCGG	ACTTAAGAGC	GTTGTTTCGG	60
ATTGTTAAGA	TTCCCGTTTG	CTGGGTTTTT	CTCCCTCAAT	CGTGCTCTCC	CGCGGCTGCC	120
TGGGGACTGG	CTCGGCCAAG	GAGGATGGAG	AGGGGGCTGC	CGTTGCTGTG	CGCCACGCTC	180
GCCCTTGCCC	TCGCCCTGGC	GGGCGCTTTC	CGCAGCGTCA	AATGTGGCGG	GACTATAAAA	240
ATTGAAAACC	CGGGGTACTT	TACATCTCCC	GGCTACCCTC	ATTCTTACCA	TCCAAGTGAG	300
AAATGTGAAT	GGCTAATCCA	AGCTCCGGAG	CCCTACCAGA	GAATCATGAT	CAACTTCAAC	360

CCACATTTTCG	ATTTGGAGGA	CAGAGACTGC	AAGTATGACT	ATGTGGAAGT	GATCGATGGA	420
GAGAATGAAG	GTGGCCGCCT	GTGGGGGAAG	TTCTGTGGGA	AGATCGCACC	TTACCTGTG	480
GTGTCTTCAG	GGCCATTTCT	CTTCATCAA	TTTGTCTCTG	ACTATGAGAC	CCACGGGGCA	540
GGATTTTCCA	TCCGCTATGA	AATCTTCAAG	AGAGGGCCCG	AATGTTCTCA	GAAGTATACA	600
GCACCTACTG	GAGTGATAAA	GTCCCTGGG	TTCCCTGAAA	AATACCCCAA	CAGCTTGGAG	660
TGCACCTACA	TCATCTTTGC	ACCAAAGATG	TCTGAGATAA	TCCTAGAGTT	TGAAAGTTTT	720
GACCTGGAGC	AAGACTCAAA	TCCTCCCGGA	GGAGTGTCT	GTCGCTATGA	CCGCTGGAG	780
ATCTGGGATG	GATTCCCTGA	AGTTGGCCCT	CACATTGGGC	GTTACTGTGG	GCAGAAAAC	840
CCTGGCCGGA	TCCGCTCCTC	TTCAGGCATT	CTATCCATGG	TCTTCTACAC	TGACAGCGCA	900
ATAGCAAAGG	AAGGTTTCTC	AGCCAACTAC	AGCGTGCTGC	AGAGCAGCAT	CTCTGAAGAT	960
TTCAAGTGTA	TGGAGGCTCT	GGGCATGGAA	TCTGGAGAGA	TCCATTCTGA	CCAGTCACT	1020
GCATCTTCCC	AGTATGGTAC	CAACTGGTCT	GTTGAGCGCT	CCCGCTGAA	CTACCTGAA	1080
AACGGGTGGA	CACCAGGAGA	GGACTCCTAC	AGGGAGTGGA	TCCAGGTGGA	CTTGGGCCTC	1140
CTGCGATTCTG	TTACTGCTGT	GGGGACACAG	GGTGCCATTT	CCAAGGAAAC	CAAGAAGAAA	1200
TATTATGTCA	AGACTTACAG	AGTAGACATC	AGCTCCAACG	GAGAGGACTG	GATCACCCCTG	1260
AAGGAGGGAA	ATAAAGCCAT	TATCTTTTTCAG	GGAAACACCA	ATCCACCGGA	TGTTGTCTTT	1320
GGAGTTTTC	CCAAACCACT	GATAACTCGA	TTTGTCCGAA	TCAAACCTGC	ATCCTGGGAA	1380
ACTGGAATAT	CTATGAGATT	TGAAGTTTAT	GGTGCAAGA	TAACAGATTA	CCCTTGCTCT	1440
GGAATGTTGG	GCATGGTGTG	TGGACTTATT	TCAGACTCCC	AGATTACAGC	ATCCAACCAA	1500
GGAGACAGGA	ACTGGATGCC	AGAAAACATC	CGCCTGGTGA	CCAGTCGAAC	CGGCTGGGCC	1560
CTGCCACCCT	CACCCACCC	ATACATCAAT	GAATGGCTCC	AAGTGGACCT	GGGAGATGAG	1620
AAGATAGTAA	GAGGTGTCT	CATTCAAGGT	GGGAAGCACC	GAGAAAACAA	AGTGTTCATG	1680
AGGAAGTTCA	AGATCGCCTA	CAGTAACAAT	GGTTCGACT	GGAAAATGAT	CATGGATGAC	1740
AGCAAGCGCA	AGGCTAAGTC	TTTTGAAGGC	AACAACAAT	ATGACACACC	TGAGCTCCGG	1800
GCCTTTATAC	CTCTCTCCAC	AAGATTATC	AGGATCTACC	CCGAGAGAGC	CACACATAGT	1860
GGCTCGGAC	TGAGGATGGA	GCTACTGGGC	TGTGAAGTAG	AAGTGCCCTAC	AGCTGGACCC	1920
ACGACACCCA	ATGGGAACCC	CGTGACGAG	TGTGACCATG	ACCAGGCCAA	CTGCCACAGT	1980
GGCAGAGTG	ATGACTTCCA	GCTCACAGGA	GGCACCCTG	TCCTGGCCAC	AGAGAAGCCA	2040
ACCATTATAG	ACAGCACCAT	CCAATCAGAG	TTCCCGACAT	ACGGTTTTAA	CTGCGAGTTT	2100
GGCTGGGGCT	CTCACAAGAC	ATTCTGCCAC	TGGGAACATG	ACAGCCACGC	GCAGCTCAGG	2160
TGGAGGGTGC	TGACCAGCAA	GACGGGGCCC	ATTACAGGACC	ACACAGGAGA	TGGCAACTTC	2220
ATCTATTCCC	AAGCTGATGA	AAATCAGAAA	GGCAAGTAG	CCCGCCTGGT	GAGCCCTGTG	2280
GTCTATTCCC	AGAGTTCTGC	CCACTGCATG	ACCTTCTGGT	ATCAGATGTC	CGGCTCTCAT	2340
GTGGGTACAC	TGAGGGTCAA	ACTGCACTAC	CAGAAGCCAG	AGGAATATGA	TCAACTGGTC	2400
TGGATGGTGG	TCGGGCACCA	AGGAGACCAC	TGGAAGGAAG	GGCGTGTCTT	GCTGCACAAA	2460
TCTCTGAAAC	TGTATCAGGT	TATTTTGTAA	GGTGAAATCG	GAAAAGGAAA	CCTCGGTGGG	2520
ATTGCTGTGG	ATGATATCAG	TATTAACAAC	CACATTCTCT	AGGAGGACTG	TGCAAAACCA	2580
ACAGACCTAG	ATAAAAAGAA	CACAGAAAT	AAAATAGATG	AAACAGGGAG	CACCCAGGA	2640
TATGAAGAAG	GGAAAGGCGA	CAAGAACATC	TCCAGGAAGC	CAGGCAATGT	GCTTAAGACC	2700
CTGGACCCCA	TCCTGATCAC	CATCATAGCC	ATGAGTGCCC	TGGGGGTGCT	CCTGGGTGCA	2760
GTCTGTGGAG	TTGTGCTGTA	CTGTGCCTGT	TGGCACAAATG	GGATGTGCGA	AAGGAACCTA	2820
TCTGCCCTGG	AGAACTATAA	CTTTGAACTT	GTGGATGGTG	TAAAGTTGAA	AAAAGATAAA	2880
CTGAACCCAC	AGAGTAATTA	CTCAGAGGCG	TGAAGGCACG	GAGCTGGAGG	GAACAAGGGA	2940
GGAGCGCGGC	AGGAGAACAG	TGGAGGCGCA	GGGACTCTGT	TACTCTGCTT	TCACTGTAAG	3000
CTGGGAAGGG	CGGGGACTCT	GTTACTCCGC	TTTCACTGTA	AGCTCGGAAG	GGCATCCGCG	3060
ATGCCATGCC	AGGCTTTTCT	CAGGAGCTTC	AATGAGCATC	ACCTACAGAC	ACAAGCAGGT	3120
GACTGCGGTA	ACAACAGGAA	TCATGTACAG	CTGCTTTTCT	TCTCTTGGTT	TCGTTTGGGT	3180
AATCAGAAGC	CAGTTGAGAC	CAAGTGTGAC	TGACTTCATG	GTTTCATCTA	CTTGGCCCCC	3240
TTTTTCTCT	CTTTCTCCTT	ACCCTGTGGT	GGATTCTTCT	CGGAACTGC	AAAATCCAA	3300
ATGCTGGCAC	TAGGCGTTGT	TCAGTGGGCT	CTTTTCGATG	ACATGTGACC	TATAGCCAG	3360
TGCCTAGAGC	ATATTAGCAT	AACCACATTT	CAGGGGACAC	CAATGTCCGC	TTTTGCATCG	3420
CTACGTGCAG	CGAGCACAGG	AAAAAGAAAA	AAAAAAGAAA	AAAAAAGAAA	AAAAAAGAAA	3471

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 922 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

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Met Glu Arg Gly Leu Pro Leu Leu Cys Ala Thr Leu Ala Leu Ala Leu
 1           5           10           15
Ala Leu Ala Gly Ala Phe Arg Ser Asp Lys Cys Gly Gly Thr Ile Lys
 20           25           30
Ile Glu Asn Pro Gly Tyr Leu Thr Ser Pro Gly Tyr Pro His Ser Tyr
 35           40           45
His Pro Ser Glu Lys Cys Glu Trp Leu Ile Gln Ala Pro Glu Pro Tyr
 50           55           60
Gln Arg Ile Met Ile Asn Phe Asn Pro His Phe Asp Leu Glu Asp Arg
 65           70           75           80
Asp Cys Lys Tyr Asp Tyr Val Glu Val Ile Asp Gly Glu Asn Glu Gly
 85           90           95
Gly Arg Leu Trp Gly Lys Phe Cys Gly Lys Ile Ala Pro Ser Pro Val
100          105          110
Val Ser Ser Gly Pro Phe Leu Phe Ile Lys Phe Val Ser Asp Tyr Glu
115          120          125
Thr His Gly Ala Gly Phe Ser Ile Arg Tyr Glu Ile Phe Lys Arg Gly
130          135          140
Pro Glu Cys Ser Gln Asn Tyr Thr Ala Pro Thr Gly Val Ile Lys Ser
145          150          155          160
Pro Gly Phe Pro Glu Lys Tyr Pro Asn Ser Leu Glu Cys Thr Tyr Ile
165          170          175
Ile Phe Ala Pro Lys Met Ser Glu Ile Ile Leu Glu Phe Glu Ser Phe
180          185          190
Asp Leu Glu Gln Asp Ser Asn Pro Pro Gly Gly Val Phe Cys Arg Tyr
195          200          205
Asp Arg Leu Glu Ile Trp Asp Gly Phe Pro Glu Val Gly Pro His Ile
210          215          220
Gly Arg Tyr Cys Gly Gln Lys Thr Pro Gly Arg Ile Arg Ser Ser Ser
225          230          235          240
Gly Ile Leu Ser Met Val Phe Tyr Thr Asp Ser Ala Ile Ala Lys Glu
245          250          255
Gly Phe Ser Ala Asn Tyr Ser Val Leu Gln Ser Ser Ile Ser Glu Asp
260          265          270
Phe Lys Cys Met Glu Ala Leu Gly Met Glu Ser Gly Glu Ile His Ser
275          280          285
Asp Gln Ile Thr Ala Ser Ser Gln Tyr Gly Thr Asn Trp Ser Val Glu
290          295          300
Arg Ser Arg Leu Asn Tyr Pro Glu Asn Gly Trp Thr Pro Gly Glu Asp
305          310          315          320
Ser Tyr Arg Glu Trp Ile Gln Val Asp Leu Gly Leu Leu Arg Phe Val
325          330          335

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Thr Ala Val Gly Thr Gln Gly Ala Ile Ser Lys Glu Thr Lys Lys Lys
 340 345 350
 Tyr Tyr Val Lys Thr Tyr Arg Val Asp Ile Ser Ser Asn Gly Glu Asp
 355 360 365
 Trp Ile Thr Leu Lys Glu Gly Asn Lys Ala Ile Ile Phe Gln Gly Asn
 370 375 380
 Thr Asn Pro Thr Asp Val Val Phe Gly Val Phe Pro Lys Pro Leu Ile
 385 390 395 400
 Thr Arg Phe Val Arg Ile Lys Pro Ala Ser Trp Glu Thr Gly Ile Ser
 405 410 415
 Met Arg Phe Glu Val Tyr Gly Cys Lys Ile Thr Asp Tyr Pro Cys Ser
 420 425 430
 Gly Met Leu Gly Met Val Ser Gly Leu Ile Ser Asp Ser Gln Ile Thr
 435 440 445
 Ala Ser Asn Gln Gly Asp Arg Asn Trp Met Pro Glu Asn Ile Arg Leu
 450 455 460
 Val Thr Ser Arg Thr Gly Trp Ala Leu Pro Pro Ser Pro His Pro Tyr
 465 470 475 480
 Ile Asn Glu Trp Leu Gln Val Asp Leu Gly Asp Glu Lys Ile Val Arg
 485 490 495
 Gly Val Ile Ile Gln Gly Gly Lys His Arg Glu Asn Lys Val Phe Met
 500 505 510
 Arg Lys Phe Lys Ile Ala Tyr Ser Asn Asn Gly Ser Asp Trp Lys Met
 515 520 525
 Ile Met Asp Asp Ser Lys Arg Lys Ala Lys Ser Phe Glu Gly Asn Asn
 530 535 540
 Asn Tyr Asp Thr Pro Glu Leu Arg Ala Phe Thr Pro Leu Ser Thr Arg
 545 550 555 560
 Phe Ile Arg Ile Tyr Pro Glu Arg Ala Thr His Ser Gly Leu Gly Leu
 565 570 575
 Arg Met Glu Leu Leu Gly Cys Glu Val Glu Val Pro Thr Ala Gly Pro
 580 585 590
 Thr Thr Pro Asn Gly Asn Pro Val Asp Glu Cys Asp Asp Gln Ala
 595 600 605
 Asn Cys His Ser Gly Thr Gly Asp Asp Phe Gln Leu Thr Gly Gly Thr
 610 615 620
 Thr Val Leu Ala Thr Glu Lys Pro Thr Ile Ile Asp Ser Thr Ile Gln
 625 630 635 640
 Ser Glu Phe Pro Thr Tyr Gly Phe Asn Cys Glu Phe Gly Trp Gly Ser
 645 650 655
 His Lys Thr Phe Cys His Trp Glu His Asp Ser His Ala Gln Leu Arg
 660 665 670
 Trp Arg Val Leu Thr Ser Lys Thr Gly Pro Ile Gln Asp His Thr Gly
 675 680 685
 Asp Gly Asn Phe Ile Tyr Ser Gln Ala Asp Glu Asn Gln Lys Gly Lys
 690 695 700
 Val Ala Arg Leu Val Ser Pro Val Val Tyr Ser Gln Ser Ser Ala His
 705 710 715 720
 Cys Met Thr Phe Trp Tyr His Met Ser Gly Ser His Val Gly Thr Leu
 725 730 735
 Arg Val Lys Leu His Tyr Gln Lys Pro Glu Glu Tyr Asp Gln Leu Val
 740 745 750
 Trp Met Val Val Gly His Gln Gly Asp His Trp Lys Glu Gly Arg Val

755	760	765
Leu Leu His Lys Ser Leu Lys Leu Tyr Gln Val Ile Phe Glu Gly Glu		
770	775	780
Ile Gly Lys Gly Asn Leu Gly Gly Ile Ala Val Asp Asp Ile Ser Ile		
785	790	795
Asn Asn His Ile Pro Gln Glu Asp Cys Ala Lys Pro Thr Asp Leu Asp		800
	805	810
Lys Lys Asn Thr Glu Ile Lys Ile Asp Glu Thr Gly Ser Thr Pro Gly		815
	820	825
Tyr Glu Glu Gly Lys Gly Asp Lys Asn Ile Ser Arg Lys Pro Gly Asn		830
	835	840
Val Leu Lys Thr Leu Asp Pro Ile Leu Ile Thr Ile Ile Ala Met Ser		845
	850	855
Ala Leu Gly Val Leu Leu Gly Ala Val Cys Gly Val Val Leu Tyr Cys		860
	865	870
Ala Cys Trp His Asn Gly Met Ser Glu Arg Asn Leu Ser Ala Leu Glu		875
	885	890
Asn Tyr Asn Phe Glu Leu Val Asp Gly Val Lys Leu Lys Lys Asp Lys		895
	900	905
Leu Asn Pro Gln Ser Asn Tyr Ser Glu Ala		910
	915	920

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 23 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

TTYGARGGNG ARATHGGNAA RGG

23

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

NAGYTCRAAR TTRATRTTYT C

21

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 8 amino acids

- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: None

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Phe Glu Gly Glu Ile Gly Lys Gly
1 5

(2) INFORMATION FOR SEQ ID NO:8:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 7 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: None

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Glu Asn Tyr Asn Phe Glu Leu
1 5

INTERNATIONAL SEARCH REPORT

International application No
PCT/US 98/14632

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 G01N33/566 G01N33/68

According to International Patent Classification(IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 G01N C07K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 95 07706 A (UNIV CALIFORNIA) 23 March 1995	1-4, 8, 11, 12, 16-18, 21, 31, 42-44, 48-51
Y	see page 38, line 29 - page 40, line 23; claims 12-18 see the whole document	5-7, 9, 10, 13-15, 19, 20, 22-30, 32-41, 45-47, 52-59

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents:

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- "&" document member of the same patent family

Date of the actual completion of the international search

16 November 1998

Date of mailing of the international search report

01/12/1998

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Authorized officer

Hoekstra, S

INTERNATIONAL SEARCH REPORT

Internatio .pplication No

PCT/US 98/14632

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	KAWAKAMI, A. ET AL.: "Developmentally regulated expression of a cell surface protein, neuropilin, in the mouse nervous system." JOURNAL OF NEUROBIOLOGY, vol. 29, 1996, page 1-17 XP002084493 see figure 1 ---	12-17, 31-41, 51-59
Y	WO 97 14424 A (UNIV CALIFORNIA) 24 April 1997 see the whole document ---	5-7,9, 10, 13-15, 19,20, 22-30, 32-41, 45-47, 52-59
Y	WO 96 30404 A (TULARIK INC) 3 October 1996 see page 17, line 19 - line 24 ---	5
P,X	CHEN H ET AL: "Neuropilin -2, a novel member of the neuropilin family, is a high affinity receptor for the semaphorins Sema E and Sema IV but not Sema III 'published erratum appears in Neuron 1997 Sep;19(3):559!." NEURON, (1997 SEP) 19 (3) 547-59. JOURNAL CODE: AN8. ISSN: 0896-6273., XP002084494 United States see the whole document ---	1-59
P,X	HE Z ET AL: "Neuropilin is a receptor for the axonal chemorepellent Semaphorin III." CELL, (1997 AUG 22) 90 (4) 739-51. JOURNAL CODE: CQ4. ISSN: 0092-8674., XP002084495 United States see the whole document ---	1-59
P,X	KOLODKIN A L ET AL: "Neuropilin is a semaphorin III receptor." CELL, (1997 AUG 22) 90 (4) 753-62. JOURNAL CODE: CQ4. ISSN: 0092-8674., XP002084496 United States see the whole document ---	1-59
A	TESSIER-LAVIGNE, MARC ET AL: "The molecular biology of axon guidance" SCIENCE (WASHINGTON, D. C.) (1996), 274(5290), 1123-1133 CODEN: SCIEAS;ISSN: 0036-8075, XP002084497 ---	1-59
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INTERNATIONAL SEARCH REPORT

Internatic Application No
PCT/US 98/14632

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	CULOTTI, JOSEPH G. ET AL: "Functions of netrins and semaphorins in axon guidance" CURR. OPIN. NEUROBIOL. (1996), 6(1), 81-8 CODEN: COPUEN;ISSN: 0959-4388, XP002084498 -----	1-59
A	KOLODKIN, ALEX L.: "Semaphorins: Mediators of repulsive growth cone guidance" TRENDS CELL BIOL. (1996), 6(1), 15-22 CODEN: TCBIEK;ISSN: 0962-8924, XP002084499 -----	1-59
A	TAKAGI, ET AL.: "Expression of adhesion molecule, neuropilin, in the developing chick nervous system." DEVELOPMENTAL BIOLOGY, vol. 170, 1995, pages 207-222, XP002084500 see page 220, right-hand column, last paragraph - page 21, left-hand column, paragraph 1 -----	1-59

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/US 98/14632

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9507706 A	23-03-1995	US 5639856 A	17-06-1997
		AU 683494 B	13-11-1997
		AU 7724094 A	03-04-1995
		CA 2171638 A	23-03-1995
		EP 0721342 A	17-07-1996
		JP 9505725 T	10-06-1997
		US 5807826 A	15-09-1998
WO 9714424 A	24-04-1997	US 5747262 A	05-05-1998
		AU 688698 B	12-03-1998
		AU 7433496 A	07-05-1997
		CA 2207505 A	24-04-1997
		EP 0802795 A	29-10-1997
WO 9630404 A	03-10-1996	US 5563039 A	08-10-1996
		AU 5424196 A	16-10-1996

m-npn-1	485	LQVDLGGDEKIVRGVVIQGGK-----HREMKVFMRKFKIAYSERNGSDWKTIMDDSKRKA	KSFEENMYDT
m-npn-2	488	LQVDLGTPTKTVKGVIIQGARGGDSITAVEARAFVRKFKVSYSLNGKDWEYIQDPRTQQT	KLFEENHYDT
h-npn-2	488	LQVDLGTPTKTVKGVIIQGARGGDSITAVEARAFVRKFKVSYSLNGKDWEYIQDPRTQQP	KLFEENHYDT
C			
m-npn-1	549	PLRTEPLSTRFIRIYPERATHSGLGLRMELGCL-----VEAPTAGPTTEPMGNPVHRC	DDQANCH
m-npn-2	550	PDIRREDPPPAQYVRYPERMSPAGIGHRLVGLGCDWTDGKPTVETLGPTTVKSEETTPPMDEDATEC	
h-npn-2	558	PDIRREDPIPAQYVRYPERMSPAGIGHRLVGLGCDWTDGKPTVETLGITVKSEETTPPTTEATEC	
SGTGDDDFQLTGOTTVLATEKPTIIDSTIOSFEPTIYGENCLFEGMOSHKTFCNMEHDSHAQLRMWVLTSTKTG	612		
	627	-----GENCSFEDDKDL-----QLPS-GENCFDFEPEET--CGWVYDHAKWLRSTWISSAMP	
	627	-----GENCSFEDDKDL-----QLPS-GENCFDFEPEET--CGWVYDHAKWLRSTWISSSP	
m-npn-1	682	PIQDHTQDCRFIYSQADEMQKGVARLVSPVVSQSSAHCHTFMYHMSGEHVGTLRVKLRYQKPEYDQL	
m-npn-2	676	HDRTFPDDKHFLLKQSDORREGQYRLISPPVHLFRSPVCHNEFQYQAMGGHVALQV--VREASQESKL	
h-npn-2	676	HDRTFPDDRHFLLRLQSDORREGQYARLISPPVHLFRSPVCHNEFQYQATGCGVALQV--VREASQESKL	
VWVVGHQGDHMKELGRVLLHKSLLKLYQVIFEGEIGKGNLGGIAYDDISIMNHISQEDCAKPTDLDKKNTE	752		
	743	LWVIREDDQGEWKHGRIILPSYDNEYQIVFEGVIGKGRSGEISQDDIRISTDVPLENCHEPISAFADYE	
	743	LWVIREDDQGEWKHGRIILPSYDNEYQIVFEGVIGKGRSGEIAIDDIRISTDVPLENCHEPISAFADYE	
IKIDETGSTPGYEGECEGDKNISRKPMVLRKTLDPILITITIIANSALGVLLGAVC-GVLLYCACWMMGMSE	822		
	813	GDWSNSSSTSGAGDPSSGKEKSW-----LYTLDPILITITIIANSALGVLLGATCAGLLLYCTCSYSGLSS	
	813	VDWSNSSSATSGCAPSTDKEKSW-----LTTLDPILITITIIANSALGVLLGATCAGLLLYCTCSYSGLSS	
cytoplasmic			
m-npn-1	891	RMLBALENYHNFELYDGVKLLKDKLNPQSNYSEA	
m-npn-2	878	RSCTTLENYHNFELYDGLK-HKVKIMNHQKCCSEA	
h-npn-2	878	RSCTTLENYHNFELYDGLK-HKVKIMNHQKCCSEA	

FIG. 3B

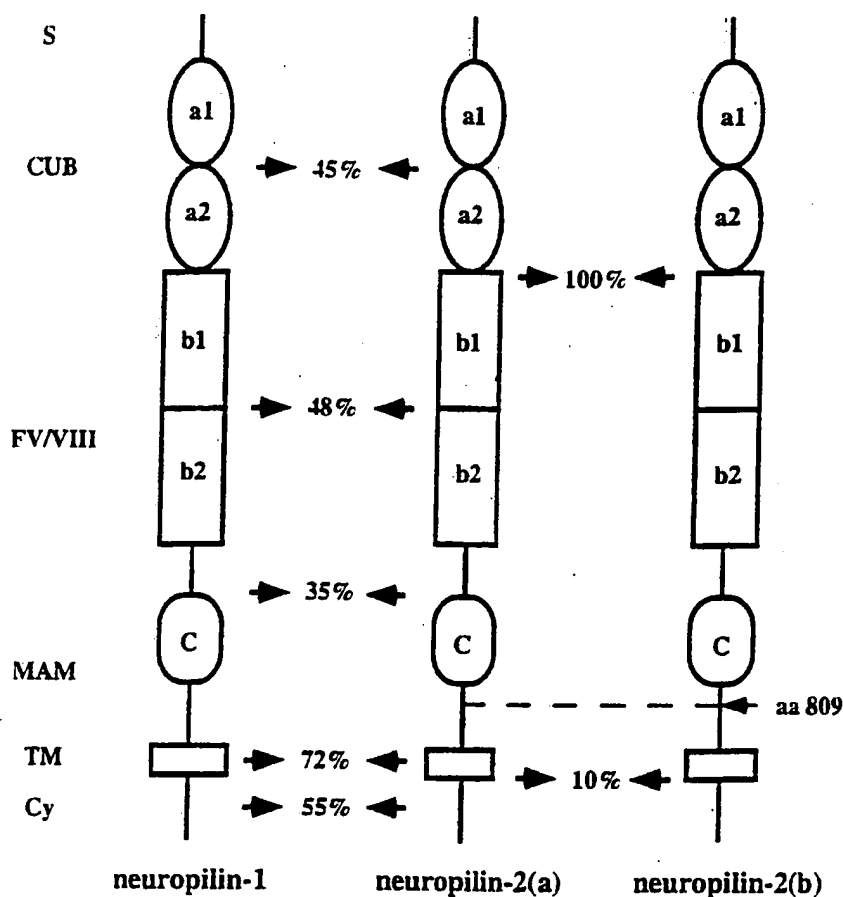


FIG 4A

m-npn-2(a0)	807	AFA ----- DEYE
m-npn-2(a5)	807	AFA GEDFK----- DEYE
m-npn-2(a17)	807	AFA -----VDIPETHGGEGYEDEIDDEYE
r-npn-2(a17)	806	AFA -----VDIPEIHGGEGYEDIIDDDYE
h-npn-2(a17)	806	AFA -----VDIPEIHEREGYEDEIDDEYE
m-npn-2(a22)	807	AFA GEDFKVDIPETHGGEGYEDEIDDEYE

FIG 4B

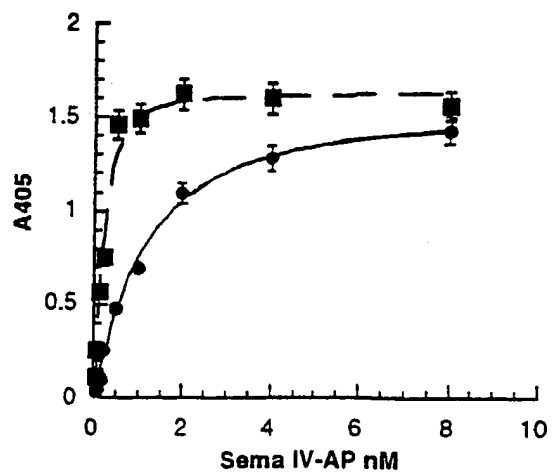
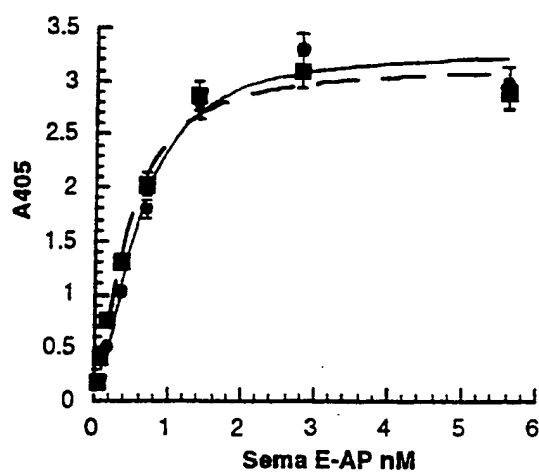
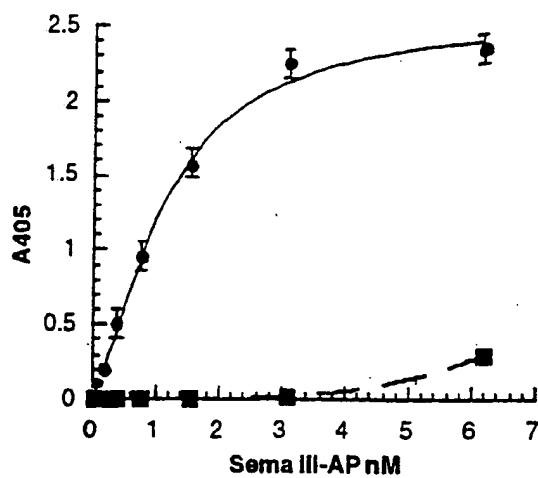
m-npn-2(b0)	807	AFA ----- GGTL
m-npn-2(b5)	807	AFA GEDFK GGTL

m-npn-2(a0)	807	AFAD EYECDWSSNSSSSSSGAGDESSGK
m-npn-2(b0)	807	AFAG---GTLP EGTPTVDIV-PMOPI
h-npn-2(b0)	81	AFAG---GVLLP GTPTVDIV-PMOPI
m-npn-2(a0)	834	EKSWIVYTLDPILITITAMSSLGVLPGA
m-npn-2(b0)	830	PAYWYYVMAAG-GAVLVLASVVLAV--
h-npn-2(b0)	104	PAYWYYVMAAG-----

m-npn-2(a0)	861	TCAGLILYCTCSYSGLSSRSCTLENY
m-npn-2(b0)	854	---VTHHRRFRMAAKKTDHSIVYKTS
h-npn-2(b0)		-----

m-npn-2(a0)	888	NFE----LYDGLKHKVRLNHOKCCSEA
m-npn-2(b0)	877	HYTNGAPMAVEPTLTIRLEOERG-SHC
h-npn-2(b0)		-----

FIG 4C



INTERNATIONAL SEARCH REPORT

 International application No.:
 PCT/US98/14290

A. CLASSIFICATION OF SUBJECT MATTER

 IPC(6) : C07K 14/435, 14/705; C12N 15/09, 15/63, 15/70, 15/81
 US CL : 530/350; 435/69.1, 320.1, 252.3, 254.11, 325; 536/23.5

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/350; 435/69.1, 320.1, 252.3, 254.11, 325; 536/23.5

 Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
 None

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, Biosis, Medline, WPI

Search Terms: semaphorin, semaphorin receptor

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X, P	HE et al. Neuropilin is a Receptor for the Axonal Chemorepellent Semaphorin III. Cell. 22 August 1997, Vol. 90, pages 739-951, especially pages 740 and 743.	1-5 and 7
Y	KAWAKAMI et al. Developmentally Regulated Expression of a Cell Surface Protein, Neuropilin, in the Mouse Nervous System. J. of Neurobiology. January 1996, Vol. 29, No. 1, pages 1-17, especially pages 2-5	1-5 and 7
X,P	KOLODKIN et al. Neuropilin is a Semaphorin III Receptor. Cell. 22 August 1997, Vol. 90, pages 753-762, especially pages 754, 755, and 758.	1-5 and 7



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
B earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*A* document member of the same patent family
O document referring to an oral disclosure, use, exhibition or other means	
P document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

11 SEPTEMBER 1998

Date of mailing of the international search report

19 OCT 1998

 Name and mailing address of the ISA/US
 Commissioner of Patents and Trademarks
 Box PCT
 Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

SALLY P. TENG

Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/14290

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	TAKAGI et al. Expression of a Cell Adhesion Molecule, Neuropilin, in the Developing Chick Nervous System. Developmental Biology. 1995, Vol. 170, pages 207-222, especially pages 209-211.	1-5, 7

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/14290

Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-5 and 7 with respect to species SR1 (SEQ ID NO: 1-6)

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/14290

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING

This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1.

Group I, claims 1-5 and 7, drawn to the semaphorin receptors and their encoding nucleic acids and a method of using the nucleic acid to make the semaphorin receptor.

Group II, claim 6, drawn to antibodies against the semaphorin receptors.

Group III, claims 8-10, drawn to a method of modulating a cell comprising a semaphorin receptor and/or a semaphorin.

The inventions listed as Groups I-III do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: The special technical features of the semaphorin receptors of Group I are their amino acid sequences and encoding nucleic acids. The special technical feature of Group II is the antibodies that bind to the receptors of Group I but does not have the amino acid sequence as the receptors of Group I. The special technical feature of Group III is a method of modulating a cell expressing a semaphorin receptor and/or a semaphorin. The special technical feature of each group is not the same or does not correspond to the special technical feature of any other group because the products of Groups I and II are structurally and functionally distinct and because the methods of Groups I and III require different reagents and method steps for achieving different goals. The groups are not linked by a special technical feature within the meaning of PCT Rule 13.2 so as to form a single inventive concept.

This application contains claims directed to more than one species of the generic invention. These species are deemed to lack Unity of Invention because they are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for more than one species to be searched, the appropriate additional search fees must be paid. The species are as follows:

- 1) SR1 (SEQ ID NO: 1-6)
- 2) SR2(a) (SEQ ID NO: 9-20)
- 3) SR2(b) (SEQ ID NO: 21-24)

The claims are deemed to correspond to the species listed above in the following manner:

The claims as they stand encompass each of the species. The claims are directed to semaphorin receptors and their encoding nucleic acids, antibodies specific for the receptors, and methods of using the receptor.

The following claims are generic: 1-10

The species listed above do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, the species lack the same or corresponding special technical features for the following reasons: Each of the species has a unique amino acid sequence and is encoded by a unique nucleic acid sequence. Thus, they are structurally distinct receptors.

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